PCT/ SE 00 / 0 1 4 4 9

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

SE00/1449

REC'D 17 AUG 2000
WIPO PCT

Intyg
C rtificat

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

(71) *Sökande* / *Applicant (s)* — *Forskarpatent i Syd AB, Lund SE*

(21) *Patentansökningsnummer* / *Patent application number* — *9902608-0*

(86) *Ingivningsdatum* / *Date of filing* — *1999-07-06*

Stockholm, 2000-08-09

För Patent- och registreringsverket
For the Patent- and Registration Office

Åsa Dahlberg

Avgift
Fee

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

Introduction

Biogenic amines were shown to be used as possible biomarkers for fish, meat, cheese, wine or beer quality and control [1, 2]. Histamine, cadaverine, and putrescine are the most known compounds from this class, their concentration being in good correlation with e.g. food freshness [3-7]. Particularly, histamine is a powerful biological active substance, can directly stimulate the heart, cause smooth muscle contraction or relaxation, stimulate both sensory and motor neurons, and control gastric acid secretion [8, 9], thus, poisoning with histamine leads to a wide variety of symptoms, i.e. urticaria, edema, abdominal cramps, headache, hypotension, palpitation and burning sensation in the mouth [10]. The toxicity of histamine is enhanced by the presence of other biogenic amines, especially because of their inhibiting effect over the histamine-metabolizing enzymes [1].

The presently used methods for determination of histamine are mostly based on chromatographic techniques [11-13], which are quite complex and require long analysis times and expensive instrumentation. Also, various methods using amine oxidases have been developed, with electrochemical [4-6, 14, 15], fluorimetric [16, 17], chemiluminometric [18], or spectrophotometric detection [19, 20]. However, these methods required either complex instrumentation [16-18] or operating at high potentials [4, 5]>were prone for biases when used in complex sample matrices.

Therefore, in this work, the possibility to amperometrically detect histamine at a low applied potential, using amine oxidase immobilized on solid graphite electrodes and two electrode designs (unmediated and mediated) is demonstrated. The performances of the

obtained biosensors were determined, compared and evaluated in a flow-injection system with electrochemical detection.

Experimental

Chemicals

Amine oxidase (AO, E.C. 1.4.3.6) from grass pea was isolated and purified according to a previously published protocol (28). Histamine dihydrochloride (Cat. no. 100340), tyramine hydrochloride (Cat. no.103173), cystamine dihydrochloride (Cat.no.100492), putrescine dihydrochloride (Cat.no. 100450), agmatine sulfate (Cat. no.100274), spermidine phosphate salt (Cat.no.102943) were from ICN Biochemicals Inc., Aurora, OH, USA. Ethylenediamine (Cat. no. 800947) was from Merck, Darmstadt, Germany. PVI_{13}-dmeOs (structure shown in figure 1) was prepared by complexing poly(1-vinylimidazole) with [osmium(4,4'-dimethylbipyridine)$_2$Cl]$^{+/2+}$ the subscription indicating that every the thirteenth mer was modified, as described elsewhere [21]. Poly (ethylene glycol) (400) diglycidyl ether (PEGDGE, Polysciences, Warrington, PA, USA, Cat. no. 08210) was used for crosslinking AO to the Os modified polymer.

Di-sodium hydrogen phosphate dihydrate (Cat. no. 1.06580) and potassium dihydrogenphosphate (Cat. no. 1.04873) both from Merck, were used for the preparation of the phosphate buffer solution, 0.1 M, pH 7.2 (PB). Water purified in a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all the solutions, if not otherwise stated.

All experiments were performed at room temperature, and before use all solutions were filtered through a 0.2 μm filter and degassed.

Electrode preparation

AO-modified graphite electrodes were prepared in two different ways:

A) type A electrodes based on a DET: rods of spectroscopic graphite (Ringsdorff Werke GmbH., Bonn, Germany, type RW001, 3.05mm diameter) were cut, and polished on fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI). After rinsing the electrode surface with water, and drying at room temperature, 6 µl of a AO stock solution 5 mg/ml was placed on the surface of the electrode.

B) type B electrodes based on a MET: first, the graphite electrodes were pretreated as for type A. Next,, 6 µl of a mixture of AO (stock solution 20 mg/ml in PB), PVI_{13}-dmeOs (stock 10 mg/ml in PB) and PEGDGE (freshly prepared and used within 15 minutes) in the desired concentrations were placed on the top of the graphite electrode. The concentration of AO was the same for both types.

If not otherwise stated, all modified electrodes were stored at 4°C for 14h in a glass beaker covered with sealing film, to prevent the rapid evaporation of the mixture. The enzyme electrodes were rinsed with PB before use.

All results presented in this paper are the mean of three equally prepared electrodes if not otherwise stated.

Instrumentation

The enzyme-modified graphite electrodes were inserted as the working electrode in a single channel flow injection system containing a manual sample injection valve (Valco Instruments Co. Inc., Houston, TX, USA) with a 50 µl injection loop.

A peristaltic pump (Alitea AB, Stockholm, Sweden) was used to pump the carrier solution at the desired flow rate through Teflon tubings (0.5 mm id.) to the flow cell. A potentiostat (Zäta-Elektronik, Lund, Sweden) was used to maintain the working potential

vs. a Ag/AgCl (0.1 M KCl) reference electrode. A platinum wire was inserted in the flow cell as a counter electrode. The resulted current was monitored with a single channel recorder (Kipp & Zonen, Delft, The Netherlands, Model BD 111).

Cyclic voltammetry experiments were carried out in a voltammetric cell, using a saturated calomel reference electrode (SCE), a platinum counter electrode and PB as electrolyte. The system was controlled by a BAS 100W Electrochemical Analyzer (Bioanalytical System Inc., West Lafayette, IN, USA), connected to a computer. The working potential was scanned between -100 and +300 mV vs. SCE. The solution was bubbled with nitrogen for 10 minutes prior and also during the experiment.

Operation stability experiments were made using an Automated Sample Injection Analyzer (Ismatec, Glattburg-Zürich, Switzerland) by injecting samples of 100 μM histamine every 2 minutes, using PB as carrier.

Results and discussion

Amine oxidases represent a class of enzymes widely distributed in mammals, plants, as well as in microorganisms [22, 23]. However, their structure, selectivity and biological functions are very different, depending on the isolation source, i.e. if in prokaryotes the presence of the enzyme allows the microorganism to use amines as a carbon source, while in both plants and animals their main role is in detoxification processes by catabolising the toxic mono- and diamines [24].

The copper-containing amine oxidases (amine oxygen oxidoreductase deaminating, copper containing; E.C. 1.4.3.6) catalyse the oxidative deamination of biogenic amines on the basis of molecular oxygen reduction, generating the corresponding aldehyde, according to the following reaction:

$$R\text{-}CH_2\text{-}NH_2 + H_2O + O_2 \rightarrow R\text{-}CHO + H_2O_2 + NH_3 \qquad (1).$$

Besides copper, the presence of an organic cofactor with a quinoide structure (topa-quinone) in the catalytic site has also been demonstrated [25]; most of the proposed reaction mechanisms have been related to the structure of this cofactor.

The kinetic mechanism of the reaction catalyzed by amine oxidases has been extensively studied since 1960s [4, 20, 26], but completely understood quite recently [27]. The substrate oxidation involves both the Cu (II) ions and the topa quinone cofactor, where the electron transfer between topa semiquinone and copper is mediated by an integrated network of water [28].

The mechanism is of ping-pong type, the reaction is occurring in three steps: the first substrate binding to the topa-quinone cofactor forms the enzyme-substrate complex, followed by its hydrolysis and the release of the aldehyde product. In the last step the reduced form of the enzyme is regenerated to its native form by oxygen, with the formation of the last two reaction products: H_2O_2 and NH_3 [24].

The principle of coupling with a secondary enzyme, i.e. a peroxidase, that converts the hydrogen peroxide formed in reaction (1), was also extensively used [17, 26]; an overview on the biosensors for biogenic amines determination presented in literature is shown in table 1.

Therefore this work focuses on the development of simple and cheap enzyme electrodes able to operate bias-free in a complex sample matrix.

DET-based electrode design

DET based electrodes are desirable since they are easy to construct and more simple, since they do not involve any additional reaction steps. However, the rate of the DET is often low [32] therefore use of coupled enzyme systems [33, 34] or MET based systems are required. In particular, redox hydrogel based electrodes were shown to display increased stability and high sensitivities [35, 36].

Cyclic voltammograms recorded for AO modified electrodes in buffer and in the presence of histamine, demonstrated a catalytic activity of the enzyme modified electrodes only in the presence of the substrate. Typical hydrodynamic voltammograms recorded for

unmodified and AO-modified electrodes (see fig.2), showed that only the AO modified electrode displayed catalytic activity, the oxidation currents starting already at -50 mV, increasing with increasing potential, and displaying a leveling of tendency above 250 mV. The half wave potential is at + 50 mV vs. Ag/AgCl (+ 270 mV vs.NHE) being in good correlation with similar values obtained by differential pulse polarography for free topa (+300mV, [28]) and the reduced form of grass-pea amine oxidase (+250mV, [28]), respectively.

Thus, the working potential was chosen to be +200 mV vs. Ag/AgCl as a compromise between the kinetics of the electrochemical reaction, the background current and the possibility of interferences which could appear at higher applied potentials, when analyzing complex matrices.

According to the reaction mechanism, the reaction pH and also the protonation state of the substrate are essential factors that can affect the enzymatic catalysis. Therefore, the effect of pH and buffer type over the response for 100 μM histamine has been studied (see fig. 3).

Histamine is a diamine, but the enzymatic conversion of only one of the two amine groups (the primary one, pKa=9.75 [37]) generates the final product, imidazole acetaldehyde. The secondary amine group is more acidic (pKa=6.04, [37]), and cannot form the enzyme-substrate complex. The observed optimum pH of about 7.0 is in good correlation both with other results presented in the literature for amine oxidases [29, 38-41], and also with the conclusion that the protonated amine is a more efficient substrate for the enzyme

[24]. In spite of higher sensitivity obtained in borate buffer, the carrier phase for flow injection experiments was chosen to be phosphate buffer, because of the noticed considerably shorter response times (results not shown).

In figure 4 is depicted the dependence of the peak current on the flow rate and the sample throughput, respectively. The decreasing peak height with increasing flow rate, indicates a limitation due to either the biocatalysed reaction or to the electrochemical process (slow electron transfer (ET) mechanism, due to slow substrate-enzyme kinetics, or the ET processes occuring at the electrode surface). On the other hand, the operating flow rate could not be to low, due to a considerable increase in response time, thus, the chosen flow rate of 0.5 ml/min, the was a compromise between the above mentioned two parameters.

MET-based electrode design

In order to improve the slow kinetics of the enzyme electrode, the use of an electrochemical mediator (PVI_{13}-dmeOs) was considered (type B electrodes).

Redox hydrogel based electrodes were shown to display improved stability due to the inclusion of the enzyme and mediator in a polymer network and hence, decreased loose of enzyme and mediator. Also, the Os redox mediator was shown to display fast kinetics and increased sensitivity of the electrodes based on this system. However, the rate of the ET was previously shown to be highly dependent on the flexibility of the redox hydrogel [33]. Therefore, these electrodes were optimized with regard to the composition of the redox hydrogel and electrode curing procedure. PVI_{13}-dmeOs and PEGDGE concentrations in

the modified enzyme electrodes were also optimized. The influence of the redox polymer-enzyme-crosslinker ratio is shown in figures 5a and 5b. The recorded signal increased with increasing crosslinker amount reading a maximum at 6.7% PEGDGE, after which the hydrogel probably become too rigid and thus caused lower diffusion rates of the electrodes through the hydrogel (5a). Similarly, increasing amount of redox polymer caused an increase in the current reaching a maximum at 27 % polymer, after which the signal decreased, indicating the electrode performance was limited by decreasing amount of enzyme. A composition formed of 66.3 % AO, 27 % PVI_{13}-dmeOs and 6.7 % PEGDGE was chosen as optimum and used in all further experiments.

Different electrode curing procedures were shown to influence the structure of the redox hydrogel, curing at elevated temperature might cause a higher cross linking, and also decrease the time needed for sensor preparation. Therefore, three curing methodes were studied namely; (i) overnight at 4°C, (ii) at room temperature for two hours and (iii) at 50°C for 10 min.

A longer adsorption time at low temperature resulted in highest sensitivity. Increasing the immobilisation time to more than 24 h at 4°C, however, did not further improve the sensitivity, rather decreased it. Curing at 50°C might deactivate the enzyme, and increasing the curing time at room temperature did not either improved the sensor characteristics (data not shown). Therefore all electrodes were cured at 4°C overnight.

The influence of the. various components of the redox hydrogel on the biosensor characteristics is shown in table 2. The increasing K_m^{app} in the presence of both PVI_{13}-dmeOs and PEGDGE demonstrated that the diffusion of the substrate was limited because

of the barrier formed by the mediator and/or cross-linking agent on the surface of the electrode, also resulting in an increased linear dynamic range. On the other hand, in the presence f the redox polycationic mediator (PVI_{13}-dmeOs), the I_{max} value was 100 % increased suggesting that the final reduction step of the topa cofactor on the electrode surface is the rate limiting step in the absence of the mediator.

The optimized biosensor design was evaluated with regard to operational stability and selectivity.

Figure 7 presents the operational stability of the both type A and B electrode using 100 μM histamine as enzyme substrate. The biosensor showed a slow decreasing tendency of the sensitivity, 10 % during the first 4 hours, 20 % decrease in 8 hours and 30 % in 10 hours.

The long term stability of the AO-modified electrode was also studied (figure 7b), calibrating the same electrodes for 10 days, keeping the electrodes at 4°C in dry conditions. No decrease of the current signal was observed for 2 days, a decrease was seen for the following 2 ones, after which the sensor was stable for the further studied 6 days.

The selectivity of both electrode designs was tested for different aromatic and aliphatic diamines. The relative responses obtained using histamine was as reference are presented in figure 8.

As seen the MET based electrodes displayed higher sensitivities for all studied diamines.

The selectivity trend, was different for the two electrode types, namely for DET-based electrode cys>his>tyr, but for MET-based electrode cys>tyr>his.

However, when a complex sample is analyzed, and in the absence of a prior separation system, the final result is a global indicator of amine content of the sample, result that could be expressed in histamine equivalents.

Conclusions

The graphite material and the cooper/quinoprotein amine oxidase required a low operating potential, thus, increasing the electrochemical selectivity of the biosensor. PVI_{13}-dmeOs modified electrodes exhibited superior characteristics to those based only on the enzyme amine oxidase; the working mechanisms of the two types of biosensors is still under investigations.

The working mechanism of the developed biosensors is still under investigations. However, the possibility of separation between the signal given by histamine, from the ones given by otherless toxic biogenic amines (e.g. cadaverine and putrescine) make the AO biosensor very suitable for analysis of histamine as a toxicity biomarker for food samples.

Acknowledgements

Authors thank to Lenka Skoupá for help with the purification of the enzyme. This work was supported by the grants 203/97/0097 from the Grant Agency and VS 96154 from the Ministry of Education, Czech Republic.

SI, EU, SJSF

Legend t the figures

FIGURE 1:

Structure of Os (4,4'-dimethylbipyridyl)$_2$Cl complexed to poly(1-vinylimidazole).

FIGURE 2:

Cyclic (a) and hydrodynamic (b) voltammograms recorded for type A electrodes for 100 µM histamine solution (A,C), and buffer solution (B), respectively, obtained using an AO-modified graphite (A, B, C), or unmodified electrode graphite (D) as working electrode. Experimental conditions as in Fig.3.

FIGURE 3:

Effect of pH and type of buffer on the response of type A electrodes for 100 µM histamine. The flow rate was 0.5 mL min^{-1}, the applied potential was 200 mV and the concentration of AO on the surface of the electrode was 5 mg/ml.

FIGURE 4:

Influence of flow rate on the response current and sample throughput, respectively. Conditions: 100 µM working solution, 5 mg/ml AO, 2 mg/ml PVI$_{13}$-dmeOs, applied potential: 200 mV vs. Ag /AgCl.

FIGURE 5:

a) Dependence of the response current on the mediator amount on the surface of the modified electrode. Conditions: PEG 0.5 mg/ml, AO 5 mg/ml.

b) Influence of the cross-linking agent on the response for histamine. PVI_{13}-dmeOs 2 mg/ml, AO 5 mg/ml.

FIGURE 6:

Effect of the immobilization conditions on the biosensor response for histamine. The flow rate was 0.5 ml/min, the applied potential was 200 mV and the components concentrations on the surface of the electrode were 66.7% AO, 26.6% PVI_{13}-dmeOs, 6.7% PEGDGE (w/w).

FIGURE 7:

Stability of the optimal AO-PVI_{13}-dmeOs-PEGDGE electrode (66.7% AO, 26.6% PVI_{13}-dmeOs, 6.7% PEGDGE (w/w%)) for injection of 100 μM histamine. Conditions: flow rate 0.5 ml/min, applied potential 200 mV vs. Ag/AgCl.

a) Operational stability. Sample throughput: 30 injections/h. The response of the first injection was considered to be 100%.

b) Storage stability. The electrode were kept in dry state at 4°C between measurements. The response in the first day was chosen as reference.

FIGURE 8:

Relative selectivity for the two types of AO biosensors. Working concentrations for different amines was 1mM, response for 1mM histamine was chosen as reference.

REFERENCES

1 J. E. Sttraton, R. W. Hutkins and S. Taylor, J. Food Protect., 54 (1991) 460-470.

2 Y. Yano, K. Yokoyama, E. Tamiya and I. Karube, Anal. Chim. Acta, 320 (1996) 269-276.

3 G. C. Chemnitius, M. Suzuki and K. Isobe, Anal. Chim. Acta, 263 (1992) 93-100.

4 K. B. Male, P. Bouvrette, J. H. T. Luong and B. F. Gibbs, J.Food Sci., 61 (1996) 1012-1016.

5 G. C. Chemnitius and U. Bilitewski, Sens. Actuators, B 32 (1996) 107-113.

6 P. Bouvrette, K. B. Male, J. H. T. Luoung and B. F. Gibbs, Enz. Microb. Technol., 20 (1997) 32-38.

7 G. Volpe and M. Mascini, Talanta, 43 (1996) 283-289.

8 A. H. Soll and A. Wollin, Gastroenter., 72 (1977) 1166.

9 S. L. Taylor, J. Y. Hui and D. E. Lyons, in E. P. Regalis (Ed.), Seafood Toxins, Washington, DC., 1984, 262, 417-430.

10 S. L. Taylor, in (Ed.), Histamine poisoning associated with fish, cheese, and other foods, World Health Organization, 1985, 1-47.

11 G. C. Yen and C. L. Hsieh, Journal of Food Science, 56 (1991) 158-160.

12 R. Draisci, S. Cavalli, L. Lucetini and A. Stacchini, Chromatographia, 35 (1993) 584-590.

13 M. T. Veciana-Nogues, T. Hernandez-Jover and A. Marine-Font, Journal of AO AC Internati nal, 78 (1995) 1045-1050.

14 C. X. Xu, S. A. M. Marzouk, V. V. Cosofret, R. P. Buck, M. R. Neuman and R. H. Sprinkle, Talanta, 44 (1997) 1625-1632.

15 R. Draisci, G. Volpe, L. Lucentini, A. Cecilia, R. Frederico and G. Palleschi, Food chemistry, 62 (1998) 225-232.

16 J. M. Hungerford and A. A. Arefyev, Anal. Chim. Acta, 261 (1992) 351-359.

17 S. Tombelli and M. Mascini, Anal. Chim. Acta, 358 (1998) 277-284.

18 M. K. Alam, M. Sasaki, T. Watanabe and K. Maeyama, Anal. Biochem., 229 (1995) 26-34.

19 S. Kubota and M. Okada, in H. U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Vol. VIII, VHC, Weinheim, 1985, pp. 566.

20 R. Stevanato, B. Mondovi, S. Sabatini and A. Rigo, Anal. Chim. Acta, 273 (1990) 391-397.

21 T. J. Ohara, R. Rajagopalan and A. Heller, Anal. Chem., 66 (1994) 2451-2457.

22 A. Rinaldi, G. Floris and A. Giartosio, in B. Mondovi (Ed.), Structure and functions of amine oxidases, Vol. CRC Press, Boca Raton, FL., 1985, pp. 51-62.

23 P. F. Knowles and D. M. Dooley, in H. Sigel and A. Sigel (Ed.), Metal ions in biological systems, Vol. 30, Dekker, New York, 1994, pp. 361-403.

24 S. W. McIntire and C. Hartmann, in V. L. Davidson (Ed.), Principles and applications of quinoproteins, Dekker, New York, 1992, 97-152.

25 S. M. Janes, D. Mu, D. Wemmer, A. J. Smith, S. Kaur, D. Maltby, A. L. Burlingame and J. P. Klinman, Science, 248 (1990) 981.

26 M. Niculescu, C. Nistor, I. Frebort, P. Pec, B. Mattiasson and E. Csoregi, submitted.

27 A. Padiglia, R. Medda, J.Z. Pedersen, A. Lorrai, P. Peč, I. Frébort and G. Floris, J. Enzyme Inhibition, 13 (1998) 311-325.

28 R. Li, J.P. Klinman and F.S. Mathews, Structure, 6 (1998) 293-307.

29 R. Gasparini, M. Scarpa, M. L. Di Paolo, R. Stevanato and A. Rigo, Bioelectrochem. Bioenerg., 25 (1991) 307-315.

30 M. Ohashi, F. Nomura, M. Suzuki, M. Otsuka, O. Adachi and N. Arakawa, J. Food. Sci., 59 (1994) 519-522.

31 R. Gasparini, M. Scarpa, F. Vianello, B. Mondovi and A. Rigo, Anal. Chim. Acta, 294 (1994) 299-304.

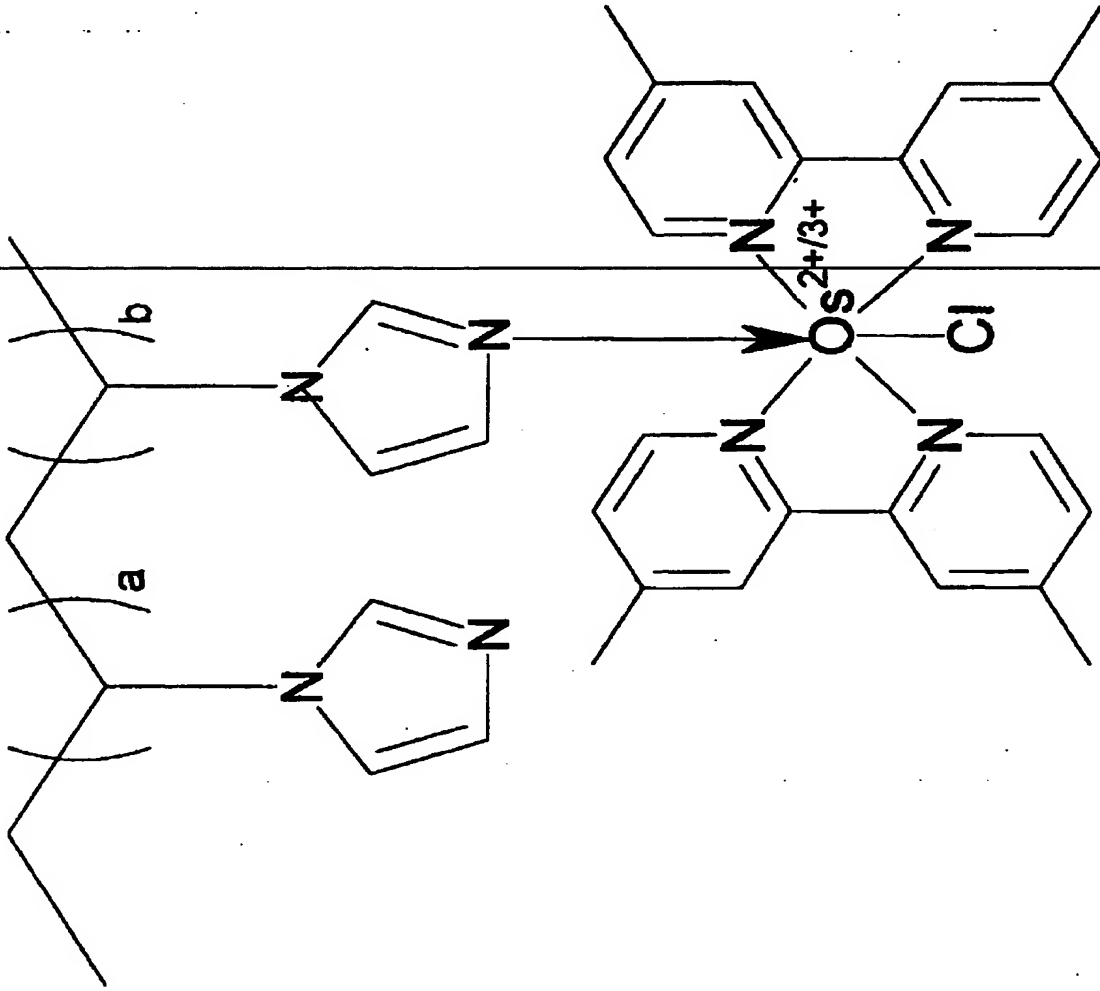
32 T. Ruzgas, E. Csoregi, J. Emneus, L. Gorton and G. Marko-Varga, Anal. Chim. Acta, 330 (1996) 123-138.

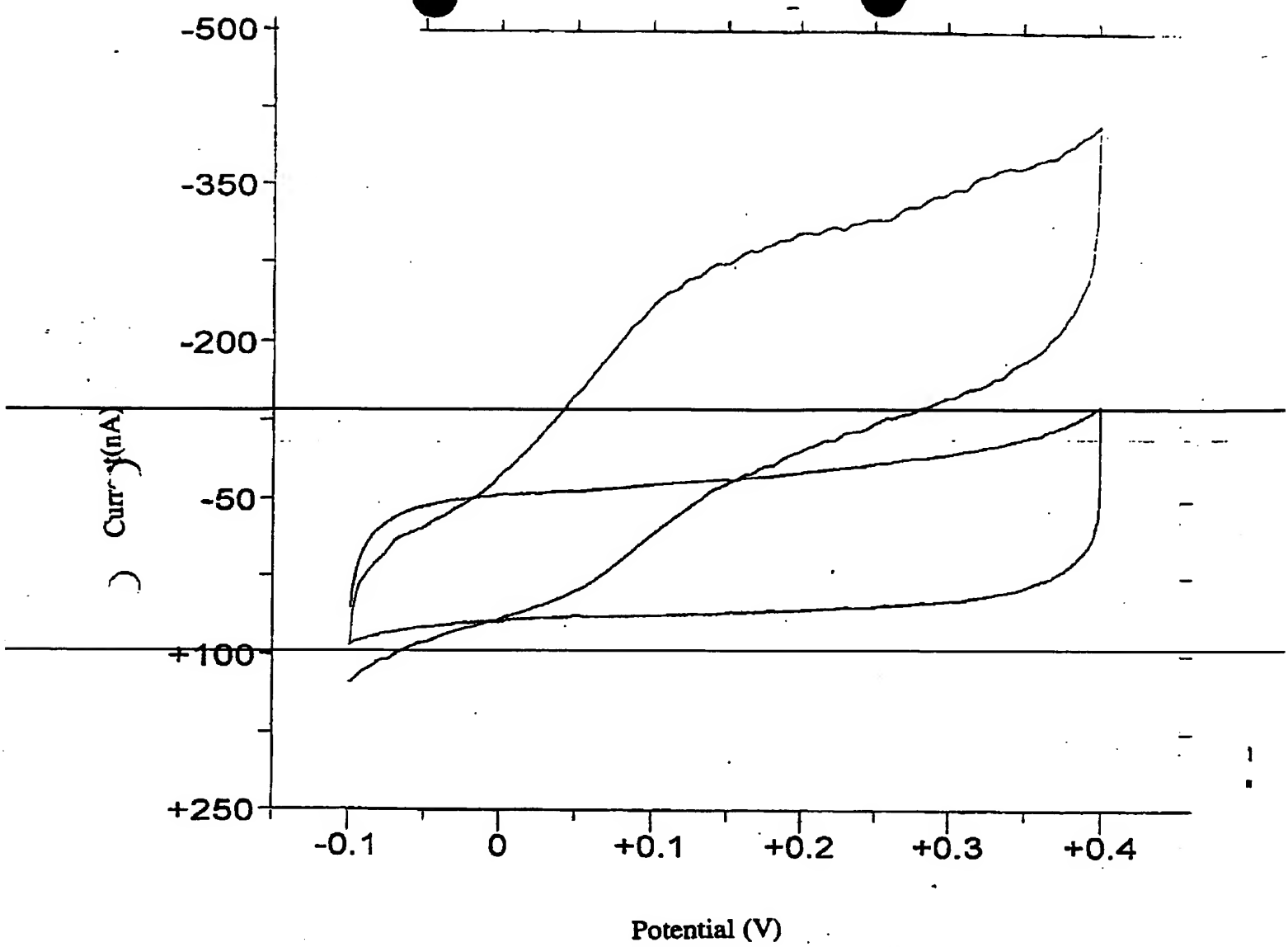
33 A. R. Vijayakumar, E. Csoregi, A. Heller and L. Gorton, Anal.Chim.Acta, 327 (1996) 223-234.

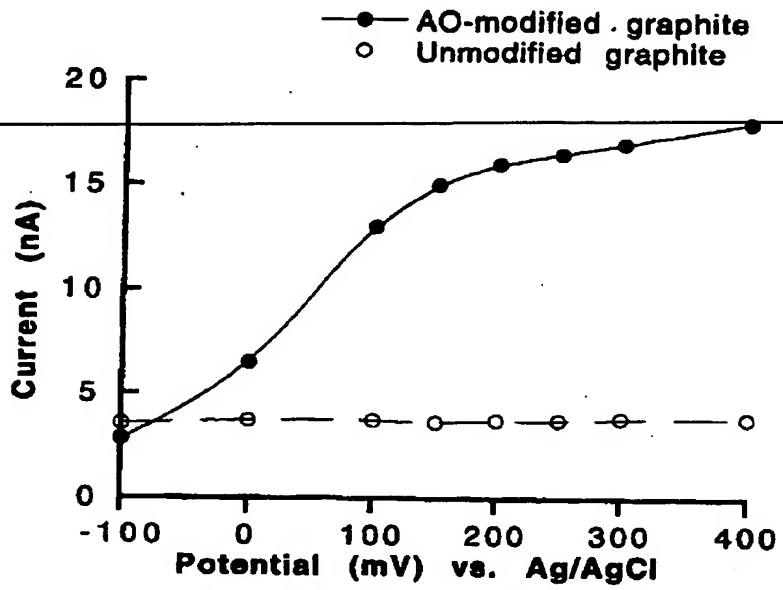
34 A. Belay, A. Collins, T. Ruzgas, P. T. Kissinger, L. Gorton and E. Csoregi, J. Pharm. Biomed. Anal., 19 (1999) 93-105.

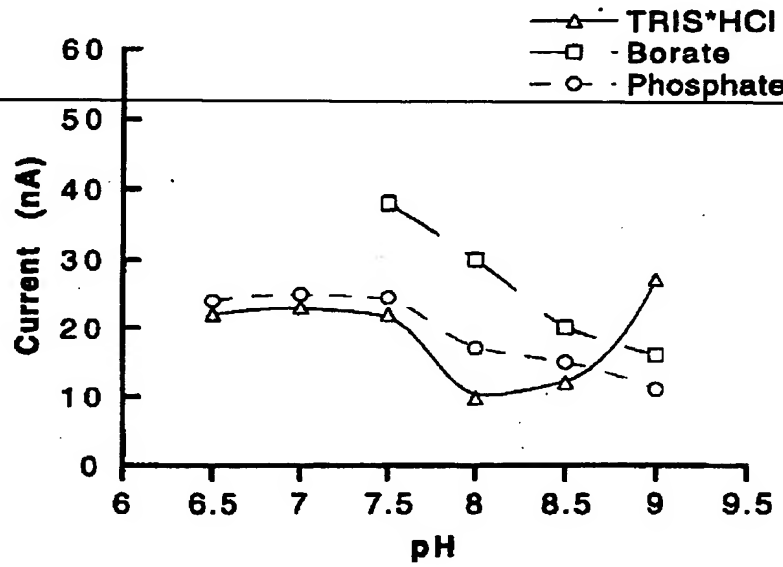
35 E. Csoregi, D. W. Schmidtke and A. Heller, Anal.Chem., 66 (1995) 2451-2457.

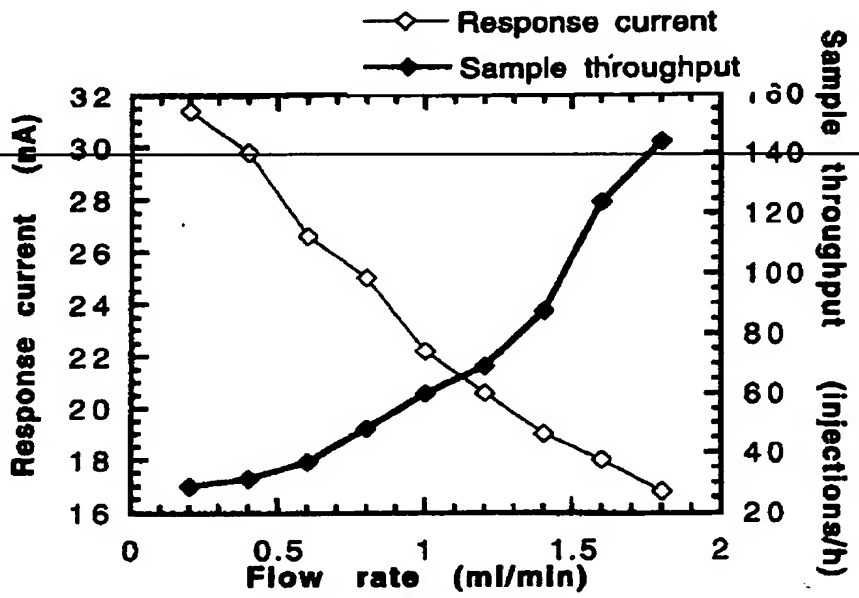
36 N. Larsson, T. Ruzgas, L. Gorton, M. Kokaia, P. T. Kissinger and E. Csoregi, Electrochim.Acta, 43 (1998) 3541-3554.

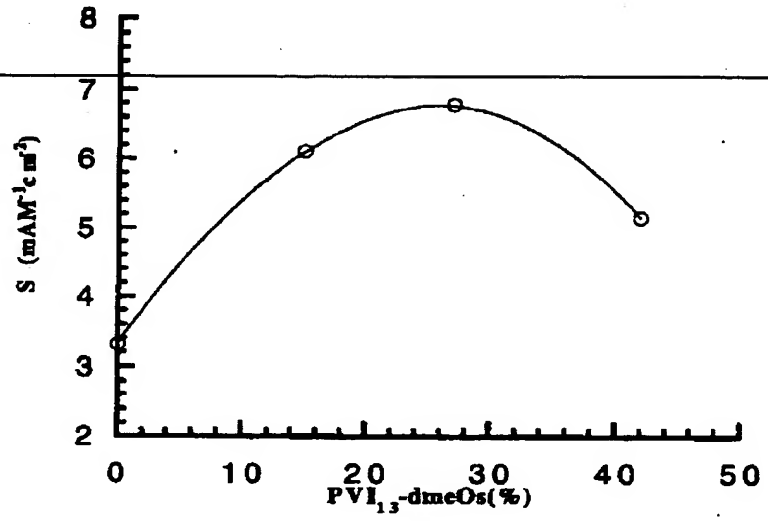


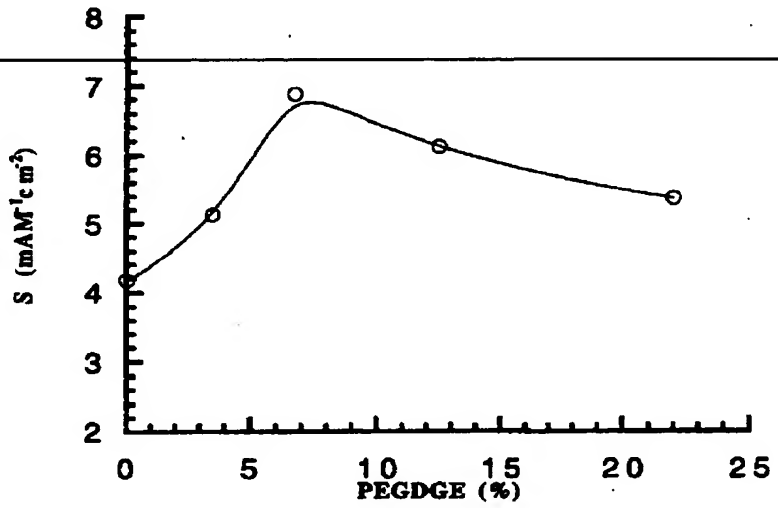


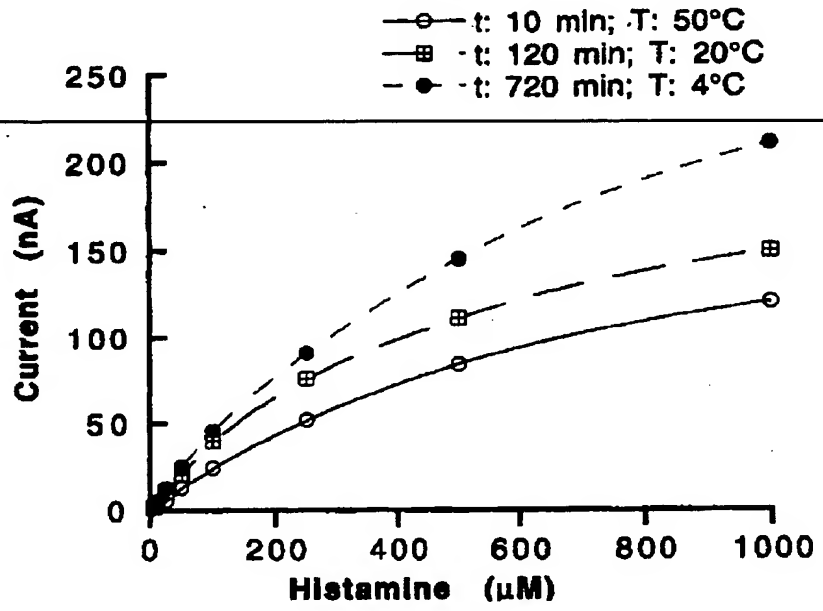


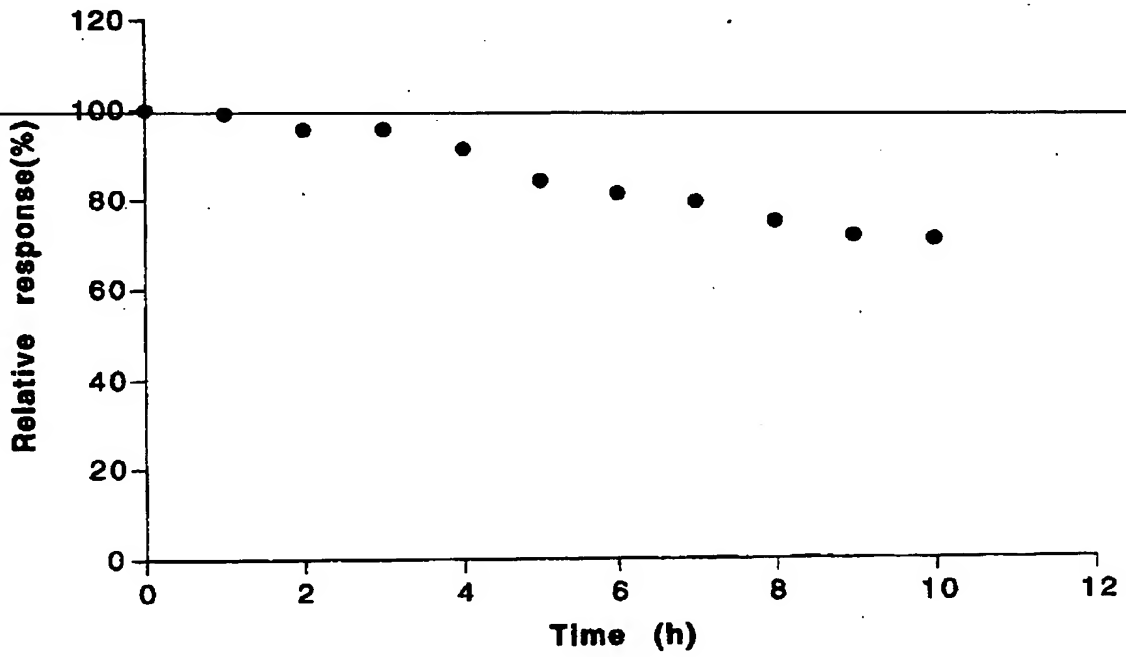


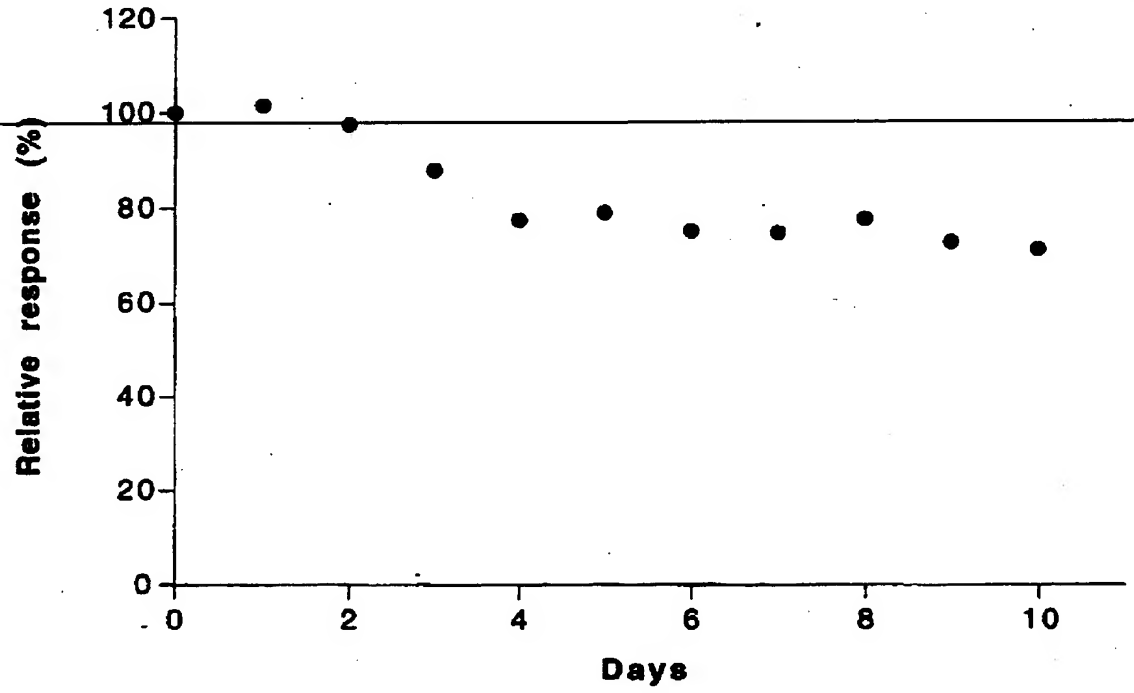












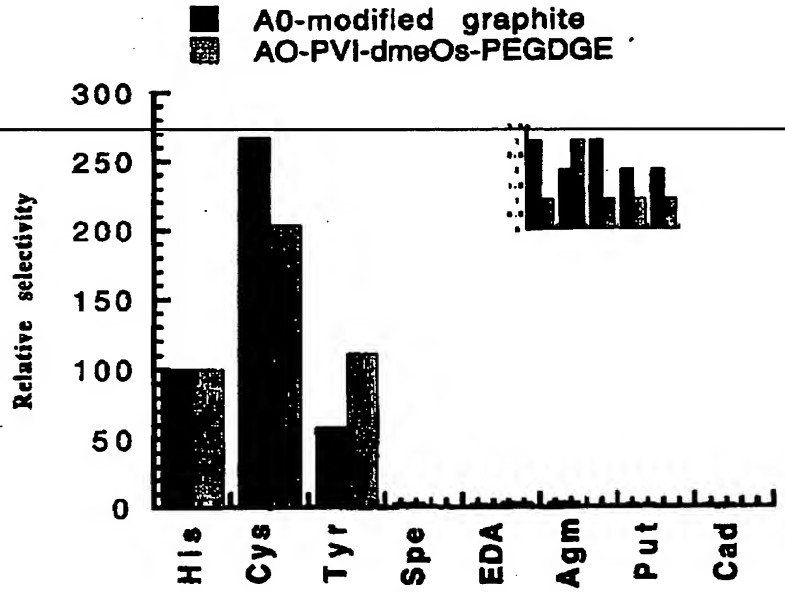


TABLE 1:

Various heretofore developed electrode configurations for the determination of biogenic amines.

BIOCHEMICAL TRANSDUCER	PHYSICAL TRANSDUCER	E (mV)	IMMOBILIZATION TECHNIQUE	LR (μM)	DL (μM)	S (mA/M)	ANALYTES	pH (BUFFER)	REF.
DAO	Pt	+650	Nylon-net Celulose acetate Polycarbonate Immobilon affinity	1-50	0.5	0.00025*	His, Put, Cad, Tyr, Spd	8.0 PB	[15]
DAO	Pt	+700	Celulose acetate Glutaraldehyde	1-100	0.6	0.025	His, Put, Cad, Spd, Benzam	7.4 P.B	[17]
DAO	Pt	+400	Immunodyne Glutaraldehyde	0-600	25	n.a	His, Put, Cad	7.2 P.B	[4]
DAO	Pt	+700	Immunodyne Glutaraldehyde	0-600	25	n.a	n.a	7.4 P.B	[6]
AO or PO	Pt paste	+600	Glutaraldehyde on silanized electrodes	0.17-20	n.a	0.93	His, Put	8.5	[5]
AO	Pt	+500	AH Sepharose BSA, Glutaraldehyde	0.5-10	0.2	0.08	Cad, Put, Spd, Spn	7.0	[29]

BIOCHEMICAL TRANSDUCER	PHYSICAL TRANSDUCER	E (mV)	IMMOBILIZATION TECHNIQUE	LR (μM)	DL (μM)	S (mA/M)	ANALYTES	pH (BUFFER)	REF.
AO	Oxygen sensor	n.a	n.a	n.a	n.a	n.a	His	8.0 P.B	[30]
PO	Pt disks 3.0 mmϕ	+300	Glutaraldehyde Nafion membrane	5-60	n.a	n.a	Put	7.0 P.B	[2]
PO	Pt	+500	n.a	0.5-300	0.5	2.16*	Put	7.2 P.B	[14]
PO	Micro planar thin-film electrode from gold	+1000	Glutaraldehyde-albumine	0.03-3	0.03	1.9	Put	8.0	[3]
DAO, HRP	Glassy carbon	0.00	Reactor	0-100	0.1	1.95	His	7.4 P:B	[17]
AO, HRP	graphite	0.00	BSA, Glutaraldehyde	1-600	5	1.83*	Put	7.4 P.B	[26]
AO, HRP	Carbon paste	+200	Fc on Sepharose	n.a	2	0.00001*	Cad, Spm	7.0 P.B	[31]

Symbols: AO-amine oxidase, DAO-diamineoxidase, PO-putrescine oxidase, HRP-horseradish peroxidase, P.B-phosphate buffer, His-histamine, Put-putrescine, Cad-cadaverine, Spd-spermidine, Spm-spermine; LR-linear range, DL-detection limit, S-sensitivity, n.a-not available, *-values what were calculated.

TABLE 2:

Response characteristics for different AO biosensors. The AO, PVI_{13}-dmeOs and PEGDGE concentrations were 5mg/ml, 2mg/ml and 0.5mg/ml, respectively.

Electrode type	K_m^{app} (μM)	I_{max} (nA)	Sensitivity ($mAM^{-1}cm^2$)	DL (μM)	DR (μM)
AO	375 ± 34	164 ± 6.5	5.99 ± 0.09	2.7	10-100
AO+PEGDGE	755 ± 38	185 ± 5.0	3.35 ± 0.05	4.5	10-150
AO+PVI_{13}-dmeOs	770 ± 14	235 ± 2.4	4.18 ± 0.02	3.7	10-150
AO+PVI_{13}-dmeOs+ PEGDGE	730 ± 33	360 ± 8.0	6.76 ± 0.05	2.2	10-200

No. 2

REDOX HYDROGEL BASED AMPEROMETRIC BIENZYME ELECTRODES FOR FISH FRESHNESS MONITORING

M. Niculescu[1], C. Nistor[2], I. Frébort[3], P. Peč[3], B. Mattiasson[1], E.Csöregi[1]*

[1]Department of Biotechnology and [2]Department of Analytical Chemistry, Lund University, P.O. Box 124, S-22 100, Lund, Sweden

[3]Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 78371 Olomouc, Czech Republic

Kolla "conclusions", jag har märkt den biten som jag tror att det [illegible] är den allra viktigaste.

— Enzymets rening → kolla ref. 32

*Corresponding author: telephone +46-46-2224274, fax +46-46-2224713, e-mail Elisabeth.Csoregi@biotek.lu.se

Abstract

This work presents the design and optimization of an electrochemical biosensor for the determination of biogenic amines (histamine, putrescine, cadaverine, tyramine, cystamine, agmatine, spermidine), commonly present in food products, and its application for monitoring of freshness in fish samples.

Amperometric bienzyme electrodes based on coupled amine oxidase, (AO) and horseradish peroxidase, (HRP) were integrated as detectors in a flow injection system for the direct assay of biogenic amines. Two different bienzyme electrodes were considered, one based on the enzymes simply adsorbed onto graphite electrodes, and one when the enzymes were crosslinked into an Os-based redox polymer. The redox hydrogel-based biosensors showed better response characteristics, i.e. sensitivity of 194.24 mA $M^{-1}cm^{-2}$ putrescine and 73.74 mA $M^{-1}cm^{-2}$ for histamine, respectively, and detection limits (calculated as three times the signal-to-noise ratio) of 0.17 μM for putrescine, and 0.33 μM for histamine.

The optimized redox hydrogel-based biosensors were evaluated in terms of stability and selectivity, and were used for the determination of total amine content in fish samples kept for 10 days in different conditions.

Key words: fish freshness, amine oxidase, horseradish peroxidase, amperometric biosensor, flow injection.

Introduction

Rapid evaluation of fish and meat quality is required in food industry, motivating a continuos search for freshness biomarkers and also the development of simple and inexpensive methods for their determination. Among these biomarkers, inositol monophosphate, hypoxanthine and xanthine, which are intermediate degradation products of nucleic acids (1, 2), and some biogenic amines, such as histamine (3-7), putrescine (8, 9) and cadaverine (8, 10), produced by microbial decarboxylation of the amino acids histidine, ornithine, and lysine, respectively, have been proposed.

The biogenic amine content of various foods has been intensively studied because of their potential toxicity (11). Histamine is the most biologically active compound from that class, affecting the normal functions of the heart, smooth muscle, motor neurons, and gastric acid secretion (12). Other biogenic amines, such as putrescine and cadaverine, may amplify the effects caused by histamine intoxication, inhibiting the enzymes involved in histamine biodegradation: diamine oxidase and histamine-N-methyl transferase (13). Numerous countries adopted maximum levels for histamine in food, especially in fish products; i.e. Italian laws fixed this level at 100 mg/Kg food (3), and similar limits have been adopted by EEC regulations (6).

Classical methods for the analysis of biogenic amines generally involve chromatographic techniques, such as gas-chromatography (14), thin layer chromatography (15), reversed-phase liquid-chromatography (15, 16), and liquid-chromatography with derivatisation techniques (17-19). However, they often require sample pre-treatment steps and skilled

operators; the relatively long analysis time and high costs make these methods not suitable for routine use.

Enzymatic determination of biogenic amines was previously carried out and represents an alternative that can solve the above mentioned problems. In this context, amperometric (9, 20), spectrophotometric (21-24), fluorimetric (25), or chemiluminimetric detection methods (14, 26) have been applied.

Amperometric electrodes using AO as the biological recognition element were also previously reported, both in single (10, 27) and coupled enzyme-based designs (15, 28). However, most of the AO biosensors required a high applied potential (>500 mV vs. Ag/AgCl) (5, 27), which can lead to higher background currents and interferences.

Therefore, in this work, a bienzymatic approach based on the enzymes amine oxidase (AO) from grass pea and horseradish peroxidase (HRP) immobilized on solid graphite has been considered, focusing on the design of a system working at potential where biases interference are minimal. First, bienzyme electrodes were prepared by simply adsorbing the two enzymes on the electrode surface using a direct electron transfer approach, but also the principle of a mediated electron transfer between the HRP and the electrode surface was exploited, by the means of a redox hydrogel formed of poly(1-vinylimidazole) complexed with $[Os(4\text{-}4'\text{-dimetylbipyridine})_2Cl]^{+/2+}$ (PVI_{13}-dmeOs) and poly(ethyleneglycol) diglycidyl ether (PEGDGE) as the crosslinking agent. The optimal biosensor design was evaluated in terms of sensitivity, lifetime and selectivity, and it was used for the analysis of fish samples stored in different conditions.

Experimental

Materials

Amine oxidase from grass pea (EC 1.4.3.6, AO) was isolated and purified according to a previously published protocol (32). Peroxidase from horseradish (EC 1.11.1.7, HRP) was purchased from Sigma Chem. Co., St. Louis, MO, USA (Cat. no. P-6782) as a lyophilized powder with a declared activity of 1100 U/mg solid. Histamine dihydrochloride (Cat. no. 100340), putrescine dihydrochloride (Cat. no. 100450), tyramine hydrochloride (Cat. no. 103173), cystamine dihydrochloride (Cat. no. 100492), agmatine sulfate (Cat. no. 100274), spermidine phosphate salt (Cat. no. 102943) were from ICN Biochemicals Inc., Aurora, OH, USA. Ethylenediamine (Cat. no. 800947) was from Merck, Darmstadt, Germany. Cadaverine dihydrochloride (Cat. no. C-8561) was purchased from Sigma. Z- and E-2-butene-1,4-diamino dihydrochloride were synthesized according to previously published protocol (29, 30). PVI_{13}-dmeOs was prepared by complexing poly(1-vinylimidazole) with [osmium(4,4'-dimethylbipyridine)$_2$Cl]$^{+/2+}$, as described elsewhere (31). Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE, Polysciences, Warrington, PA, USA, Cat. no. 08210) was used for crosslink both AO and HRP to the osmium complexed polycation. Di-sodium hydrogen phosphate dihydrate and potassium dihydrogenphosphate, purchased from Merck, (Cat. no. 1.06580 and no. 1.04873, respectively), and water purified in a Milli-Q system (Millipore, Bedford, MA, USA) were utilized to prepare the phosphate buffer 0.1 M, pH 7.2 (PB), used as the carrier solution and supporting electrolyte in the flow injection experiments, if not otherwise stated. Hydrogen peroxide, 35% wt. water solution was from Across Organics, NJ, USA.

All experiments were performed at room temperature. All solutions were daily prepared using PB as solvent, if not otherwise stated, and were filtered through 0.2 μm filters and degassed before use.

Biosensor preparation

Bienzyme (AO and HRP) modified graphite electrodes were prepared as follows: first, rods of spectroscopic graphite (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter) were cut, and polished on a wet fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights,MI). Next the electrode surface was rinsed with water, dried at room temperature before coating with enzymes. Three different electrode types were prepared:

Type I electrodes: were prepared by placing 6 μl of a premixed solution containing various amounts of AO (stock 20 mg/ml in PB) and HRP (stock 10 mg/ml in PB) on the graphite electrode (direct electron transfer approach).

Type II electrodes: 6 μl of a mixture formed of AO (stock solution 20 mg/ml in PB), HRP (stock 10 mg/ml in PB), PVI_{13}-dmeOs (stock 10 mg/ml in PB) and PEGDGE (5 mg/ml freshly prepared and used within 15 min) in different w/w (%) ratios were placed on the top of the graphite electrode (one layer electrodes).

Type III electrodes: were prepared using a sequential coating procedure; the four components of the mixture described above were separated in two groups; 6 μl of the first one were initially added on the graphite, and, after its drying, the second layer formed by 6

µl of the other group of components was placed over the first one (two layer electrodes), as following:

type III a - first a premixed solution 6 µl of HRP, PVI_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, the electrodes were dried for 1 hour before coating with 6 µl of AO (see table III).

type III b - first a solution of 6 µl of AO was placed on the top of the electrode. After drying for 1 hour, the electrodes were coated with 6 µl of a premixed solution of HRP, PVI_{13}-dmeOs, and PEGDGE.

type III c - in the first step, a drop of HRP solution (6 µl) was placed on the top of the electrode and, after its drying, a second layer containing 6 µl of a pre mixed solution of AO, PVI_{13}-dmeOs, and PEGDGE was added.

type III d - first a pre mixed solution of 6 µl of AO, PVI_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, electrodes were dried for 1 hour before coating with 6 µl of HRP.

If not otherwise stated, all modified electrodes were stored at 4° C for 14 h in a glass beaker and were rinsed with PB before use.

All results presented in this paper are means of at least three equally prepared electrodes.

Instrumentation

The bienzyme-modified graphite electrodes were inserted as the working electrode in a single channel flow injection system containing a manual sample injection valve (Valco

Instruments Co. Inc., Houston, TX, USA) with a 50 µl injection loop and a three electrodes cell of the wall-jet type.

A peristaltic pump (Alitea AB, Stockholm, Sweden) was used to pump the carrier solution at desired flow rates through Teflon tubings (0.5 mm i.d.) to the flow cell. A potentiostat (Zäta-Electronik, Lund, Sweden) maintained the constant potential between the working and the Ag/AgCl (0.1 M KCl) reference electrode. A platinum wire was used as the counter electrode. The response current was monitored with a single channel recorder (Kipp & Zonen, Delft, The Netherlands, Model BD 111).

Operational stability experiments were made using an Automated Sample Injection Analyser (Ismatec, Glattburg-Zürich, Switzerland) by injecting samples of 100 µM histamine and 50 µM putrescine, respectively, with a sample through-put of 30 injections/h, using PB as the carrier solution at a flow rate of 0.5 ml/min.

Sample preparation

The frozen fish-muscle samples (turbot - *Psetta maxima*) were kindly provided by Dr. Gunilla Önning, Department of Applied Nutrition and Food Chemistry, Lund University, Sweden. Triplets of 1.0 g fish samples kept in different conditions were homogenized in 10 ml of PB. The homogenate was centrifuged at 13000 g for 60 min. at 4° C. The supernatant was separated and immediately analyzed by direct injection into the flow system.

Results and Discussion

Amine oxidases represent a class of enzymes with a ubiquitous distribution in mammals, plants and in micro-organisms (32, 33). However, the structure, selectivity and biological functions are very different, depending on the isolation source. Grass-pea amine oxidase (46), used during this work, is a copper-containing AO, which besides the metal ions contains also an organic cofactor with a quinoide structure (topa-quinone) in its catalytic site (34); most of the proposed reaction mechanisms have been related to the structure of this cofactor. In most of the developed methods an amine oxidase is used, the enzyme converting the analyte to a corresponding aldehyde with NH_3 and H_2O_2 release, according to reaction (1):

$$R\text{-}CH_2\text{-}NH_2 + H_2O_2 + O_2 \rightarrow R\text{-}CHO + H_2O_2 + NH_3 \quad (1)$$

Both, the oxygen consumption (7, 35) or hydrogen peroxide formation (3, 4) have been used for monitoring of biogenic amines on the basis of the above mentioned reaction.

Also, the principle of coupling AO reaction with one catalyzed by a secondary enzyme, i.e. a peroxidase (15, 28), has also been exploited. A biosensor based only on the enzyme amine oxidase immobilized on a graphite electrode has recently been described (36);however, the necessity of a high applied potential (+200 mV vs. Ag/AgCl) might cause increased interferences in complex matrices (e.g., food samples).

The combination of peroxidases with hydrogen peroxide-producing oxidases for the development of amperometric biosensors has been extensively used in the past years (37, 38). The possibility of direct electron transfer between the peroxidase and an appropriate

electrode at very low applied potentials (around 0 mV vs. SCE (39)) makes that type of biosensors suitable for applications in real matrices.

The bienzyme approaches concepted in this w rk were based on the direct coupling of AO and HRP simply immobilized on the electrode surface (type I) and entrapped into a redox hydrogel (type II and III). In all the configurations, amine oxidase first converts the amine substrate (e.g., histamine) to an aldehyde product, the active form of the enzyme being recovered by oxidation of the organic cofactor in presence of molecular oxygen (see fig. 1). The hydrogen peroxide formed during the first reaction is subsequently reduced to water by the action of peroxidase; the native form of the second enzyme is re-made either by direct reduction of its heme cofactor on the electrode surface (see fig. 1a, type I biosensors), or by receiving electrons from a mediator, maintained in its reduced form by the potential applied on the graphite electrode (see fig. 1b, type II biosensors).

Optimization steps

The bienzyme electrodes were optimized with regard to several parameters, e.g., working potential, the flow rate, influence of various enzyme ratios and electrode coating procedure.

Hydrodynamic voltammograms were made for 100 µM histamine using an AO-HRP-modified electrode in order to establish the optimal working potential. The voltammogram, together with the ratio between the response and the background current obtained in the same conditions, respectively, are shown in figure 2. Even the response of the biosensor drastically increased when the applied potential was below -100 mV, the background current evolution had a similar shape that demonstrates the possible oxygen

electroreduction interference with the biosensing process. A potential of -50 mV vs. Ag/AgCl was chosen for further experiments as a compromise between the response and the background current.

The carrier flow rate influence on the biosensor response for histamine was studied, the results being presented in figure 3. The decrease in peak height with the increase in flow rate demonstrates a limitation due either to the bioconversion of the amine substrate by AO or to the reduction of H_2O_2 by the direct electron transfer between HRP and graphite electrode. Even the second mentioned reaction is known to be a slow process (40), one of our recent studies has previously shown that the reaction catalyzed by AO is also rate limiting in another biosensor configuration (36).

The optimal working flow rate was chosen to be 0.5 ml/min, as a compromise between the biosensor kinetics and its sample throughput, respectively.

In order to achieve an effective electron transfer (ET) all electrode types were optimized with regard to the **ratio of the** used **enzymes** (type I), **composition of the redox hydrogel** (type II) and influence of **electrode coating procedures** (type III a - III d). Table 1 shows the kinetic parameters and the main biosensor characteristics obtained for type I electrodes at different ratios of AO:HRP.

The increasing tendency of the apparent Michaelis constant with the amount of immobilized HRP was attributed to an increase in the thickness of the total protein loading on the electrode surface, that has as an effect the reduction of the analytes diffusion rate in the film; the maximum current, as well as the biosensors sensitivity trend show that the best combination is the one containing 80% AO and 20% HRP (w/w), which has been

considered for the further experiments. The dynamic range for all the studied biosensors type I was 1-100 μM for both histamine and putrescine.

In order to improve the electron transfer kinetics between HRP and the graphite electrode, as well as the biosensor stability, an Os-based redox polymer was tested as electrochemical mediator and PEGDGE as a cross-linking agent. Redox hydrogels have been previously shown to represent an effective matrix for enzyme immobilized resulting in increased stability and the enhanced rate of the ET (41-44). It is known however that the rate of the ET is highly influenced by the composition of the redox hydrogel, as well as by the kinetics of the used enzymes. Therefore various biosensor designs were considered in order to find the optimal electrode structure displaying the most efficient ET.

The redox hydrogel based biosensors were first examined in order to determine the influence of the redox polycation and the crosslinking agent. Table II shows the obtained results. If the diffusional barrier increased with the number of components added on the electrode surface, a tendency reflected in the apparent Michaelis constants (increase with about 171 % for histamine and 125 % for putrescine), introduction of the electrochemical mediator causes a considerable improvement in bioelectrocatalytic efficiency, as seen from increase in the I_{max} (with 262 % for histamine and 141 % for putrescine) and sensitivity values (with 33 % for histamine and 7 % for putrescine). The hydrogen peroxide sensitivity remains practically unchanged, confirming the previously reported results for other bienzyme hydrogel electrodes (45). The detection limit and also the dynamic range for the studied analytes have also been improved in the case of type II electrodes. The combination containing the two enzymes, and both the mediator and cross-linking agent

was chosen for further experiments because also of its improved stability (results not shown).

The effect of the **coating procedure** for the type II - III biosensors was also studied. Besides coating with a premixed solutions of all four components, different possibilities of sequential coatings of the electrode surface were also studied (see table III). It was previously demonstrated that both HRP and AO can be electrically wired to the redox polymer, and thus cause a partial short-circuit (36, 45) when all components are mixed together. This assumption was confirmed for the main substrate (putrescine) for which an increase in sensitivity of about 30 % was observed for the two layers electrodes (type III), compared to the one layer ones (type II). No considerable change was observed for the other substrate histamine, the slight decrease in sensitivity being not representative considering the differences of about 10-15 % in electrode preparation. Clearly, the less sensitive electrode configuration is represented by III d type electrodes for which the bias currents due to the wiring of AO are the most explicit. Considering the simplicity of electrode preparation and the small differences in the electrode characteristics between type II and type III electrodes, type II was chosen as optimal electrode design.

Characteristics and applications

Type II biosensors were further characterized with regard to **selectivity, operational and storage stability**.

Figure 4 shows the relative selectivity for different AO substrates, using histamine as a reference compound, since is considered to be the biomarker of major interest. As seen, the response for aliphatic amines is generally higher than for the aromatic ones; also, the

type II biosensor give, with two exceptions, improved results than type I in relation with histamine, probably due to the better electron transfer kinetics.

Figure 5a shows the operational stability of the biosensor using both putrescine and histamine as substrates. As seen, the response current of the bienzymatic enzyme electrodes decreased with about 30% for histamine, and with about 50% for putrescine, after 10 hours of continuous operation with a sample through-put of 30 injections/hour. The storage stability was good, a decrease of only about 10% from the initial response for both histamine and putrescine being observed after 10 days of storage (see fig. 5b).

The optimized biosensor was considered for **monitoring biogenic amines in real samples**. As a major drawback, the differentiation between the signals given by different amines is not possible, but only the total amine content of a sample could be determined. Fish-muscle samples, kept both at 4° C and 25° C for 10 days, were analyzed after extraction in PB by direct injection in the flow system. The total amine content expressed in histamine equivalents is presented in fig. 6.

The maximum accepted limit for total amines concentration in food products is 100 to 200 mg/Kg sample, and a concentration of 1000 mg/Kg is considered to be toxic(6). As seen, after 3 days of storage at room temperature, the fish become improper to consume, while even after 10 days of storage at 4° C they are not major changes in the total amine concentration.

C nclusi ns

The present work shows the development, optimizations and applications of a bienzyme electrode for determination of biogenic amines, both in simple and mediated forms.

The optimized redox hydrogel incorporated bienzyme electrodes were characterized by high sensitivity, excellent operational stability, fast response time, and low detection limit, making them very promising for determination of food quality.

As previously demonstrated, a biosensor with only AO immobilized on a graphite electrode is selective for histamine, cystamine and tyramine towards cadaverine and putrescine. Using a combination of the two developed biosensors the separation of the signals given by the mentioned amines would be possible. The short response times, low detection limits for amines, as well as the good stability make the developed biosensor suitable for routine use in analysis of food products.

Acknowledgments

This work was supported by the grants 203/97/0097 from the Grant Agency and VS 96154 from the Ministry of Education, Czech Republic, and EC Contract IC15-CT98-0910. The Swedish Institute is acknowledged by Mihaela Niculescu for financial support.

LEGEND TO THE FIGURES

FIGURE 1:

Electron transfer pathways for the two studied bienzyme electrode designs using histamine as model substrate:

a. direct electron transfer;

b. mediated electron transfer.

Symbols: AO_{ox}, AO_{red}, HRP_{ox}, HRP_{red} are the oxidized and reduced forms of the mentioned enzymes, respectively; TOPA and TOPAQ are trihydroxyphenylalanine and its quinonic form, respectively, the reduced and oxidized states of the AO cofactor; Fe^{3+} is the oxidation state of the Fe ion in the native peroxidase, Fe^{4+}=O is an oxyferryl iron, and P^{+} - a cation radical of the porphyrin ring or protein chain (40), last two presented in the oxided (inactive) form of HRP.

FIGURE 2:

Hydrodynamic voltammogram for 100 μM histamine using a AO-HRP modified graphite electrode (I), the background current obtained in the same conditions (I_0), and the ratio between them (I/I_0), respectively. Conditions: electrodes type I (direct electron transfer approach), AO:HRP 1:1 (w/w), flow rate 0.5 ml/min.

FIGURE 3:

The effect of the flow rate on the response current and sample throughput of the type I biosensors, respectively. Conditions: injections of 100 μM histamine, AO:HRP 1:1 (w/w), applied potential: -50 mV vs. Ag/AgCl.

FIGURE 4:

The relative selectivity for different amine oxidase substrates, using histamine as reference compound. Symbols: His - histamine; Cys - cystamine; Tyr - tyramine; Spr - spermidine; EDA - ethylenediamine; Agm - agmatine; Put - putrescine; Cad - cadaverine; Z-Ab - Z-1,4-diamino-2-butene; E-Ab - E-1,4-diamino-2-butene.

FIGURE 5:

Stability of the optimal AO-HRP-PVI13-dmeOs-PEGDGE electrode (type II. a) for injection of 100 μM histamine and 50 μM putrescine, respectively. Conditions: flow rate 0.5 ml/min, applied potential -50 mV vs. Ag/AgCl.

a. Operational stability. Sample throughput: 30 injections/h. The response of the first injection was considered to be 100%.

b. Storage stability. The electrodes were kept in dry state at 4 C between measurements. The response in the first day was chosen as reference.

FIGURE 6:

Determination of fish freshness using AO-HRP-PVI_{13}-dmeOs-PEGDGE biosensor (type II a). The total amine concentration is expressed in histamine equivalent units.

REFERENCES

(1) Volpe, G.; Mascini, M. *Talanta* **1996,** *43*, 283-289.

(2) Gruger, E. *J. Agric. Food Chem.* **1972,** *20*.

(3) Tombelli, S.; Mascini, M. *Anal. Chim. Acta* **1998,** *358*, 277-284.

(4) Male, K. B.; Bouvrette, P.; Luong, J. H. T.; Gibbs, B. F. *J.Food Sci.* **1996,** *61*, 1012-1016.

(5) Bouvrette, P.; Male, K. B.; Luoung, J. H. T.; Gibbs, B. F. *Enz. Microb. Technol.* **1997,** *20*, 32-38.

(6) Chemnitius, G. C.; Bilitewski, U. *Sens. Actuators* **1996,** *B 32*, 107-113.

(7) Ohashi, M.; Nomura, F.; Suzuki, M.; Otsuka, M.; Adachi, O.; Arakawa, N. *J. Food. Sci.* **1994,** *59*, 519-522.

(8) Yano, Y.; Yokoyama, K.; Tamiya, E.; Karube, I. *Anal. Chim. Acta* **1996,** *320*, 269-276.

(9) Chemnitius, G. C.; Suzuki, M.; Isobe, K. *Anal. Chim. Acta* **1992,** *263*, 93- 100.

(10) Gasparini, R.; Scarpa, M.; Di Paolo, M. L.; Stevanato, R.; Rigo, A. *Bioelectrochem. Bioenerg.* **1991,** *25*, 307-315.

(11) Taylor, S. L. *Crit. Rev. Toxicol.* **1986,** *17*, 91-128.

(12) Sttraton, J. E.; Hutkins, R. W.; Taylor, S. *J. Food Protect.* **1991,** *54*, 460- 470.

(13) Hui, J. Y.; Taylor, S. L. *Toxicol. Appl. Pharmacol.* **1985,** *8*, 241-249.

(14) Bachrach, U.; Plesser, Y. M. *Anal. Biochem.* **1986,** *152*, 423-431.

(15) Yang, X.; Rechnitz, G. A. *Electroanalysis* **1995,** *7*, 105-108.

(16) Hauschild, M. Z. *J. Chromatogr.* **1993,** *630*, 397-401.

(17) Hui, J. Y.; Taylor, S. L. *J. AOAC Int.* **1983,** *66,* 853-857.

(18) Yen, G. C.; Hsieh, C. L. *J. Food Sci.* **1991,** *56,* 158-160.

(19) Veciana-Nogues, M. T.; Hernandez-Jover, T.; Marine-Font, A. *J. AOAC Int.* **1995,** *78,* 1045-1050.

(20) Xu, C. X.; Marzouk, S. A. M.; Cosofret, V. V.; Buck, R. P.; Neuman, M. R.; Sprinkle, R. H. *Talanta* **1997,** *44,* 1625-1632.

(21) Matsumoto, T.; Suzuki, O.; Katsumata, Y.; Oya, M.; Suzuki, T.; Nimura, Y.; Hattori, T. *J. Cancer Res. Clin. Oncol.* **1981,** *100,* 73-84.

(22) Stevanato, R.; Mondovi, B.; Sabatini, S.; Rigo, A. *Anal. Chim. Acta* **1990,** *273,* 391-397.

(23) Rinaldi, A. C.; Sanjust, E.; Rescigno, A.; Finazziagro, A.; Rinaldi, A. *Biochem. Mol. Biol. Int.* **1994,** *34,* 699-704.

(24) Toul, Z.; Macholán, L. *Collect. Czech. Chem. Commun.* **1975,** *40,* 2208-2217.

(25) Hungerford, J. M.; Arefyev, A. A. *Anal. Chim. Acta* **1992,** *261,* 351-359.

(26) Alam, M. K.; Sasaki, M.; Watanabe, T.; Maeyama, K. *Anal. Biochem.* **1995,** *229,* 26-34.

(27) Draisci, R.; Volpe, G.; Lucentini, L.; Cecilia, A.; Frederico, R.; Palleschi, G. *Food Chem.* **1998,** *62,* 225-232.

(28) Gasparini, R.; Scarpa, M.; Vianello, F.; Mondovi, B.; Rigo, A. *Anal. Chim. Acta* **1994,** *294,* 299-304.

(29) Peč, P.; Chudý, J.; Macholán, L. *Biológia (Bratislava)* **1991,** *46,* 665-672.

(30) Macholán, L.; Hubálek, F.; Šubová, H. *Collect Czech. Chem. Commun.* **1975,** *40,* 1247-1265.

(31) Ohara, T. J.; Rajagopalan, R.; Heller, A. *Anal. Chem.* **1994,** *66,* 2451-2457.

(32) Šebela, M., Luhová, L., Frébort, I., Faulhammer, H.G., Hirota, S., Zajoncová, L., Stužka, V., Peč, P., *Phytochem. Anal.* **1998,** *9, 211-222.*

(33) Knowles, P. F.; Dooley, D. M. *Metal ions in biological systems*; Dekker: New York, 1994.

(34) Janes, S. M.; Mu, D.; Wemmer, D.; Smith, A. J.; Kaur, S.; Maltby, D.; Burlingame, A. L.; Klinman, J. P. *Science* **1990,** *248,* 981.

(35) Karube, I.; Satoh, I.; Araki, Y.; Suzuki, S. *Enzyme and Microbial Technology* **1980,** *2,* 117-120.

(36) Niculescu, M.; Frébort, I.; Peč, P.; Galuszka, P.; Mattiasson, B.; Csöregi, E. *submitted.*

(37) Vijayakumar, A. R.; Csöregi, E.; Heller, A.; Gorton, L. *Anal.Chim.Acta* **1996,** *327,* 223-234.

(38) Ghobadi, S.; Csöregi, E.; Marko-Varga, G.; Gorton, L. *Current Separations* **1996,** *14,* 94-102.

(39) Marko-Varga, G.; Emnéus, J.; Gorton, L.; Ruzgas, T. *Trends Anal. Chem.* **1995,** *14,* 319-328.

(40) Ruzgas, T.; Csöregi, E.; Emnéus, J.; Gorton, L.; Marko-Varga, G. *Anal. Chim. Acta* **1996,** *330,* 123-138.

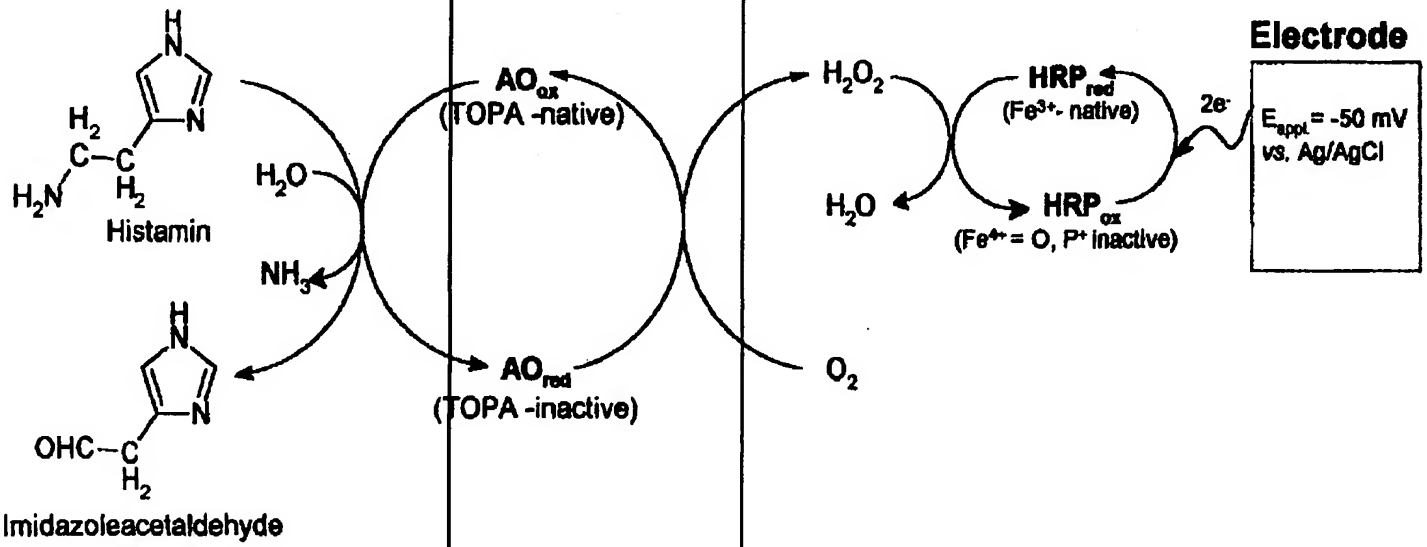
(41) Heller, A. *J. Phys. Chem.* **1992,** *96,* 3579-3587.

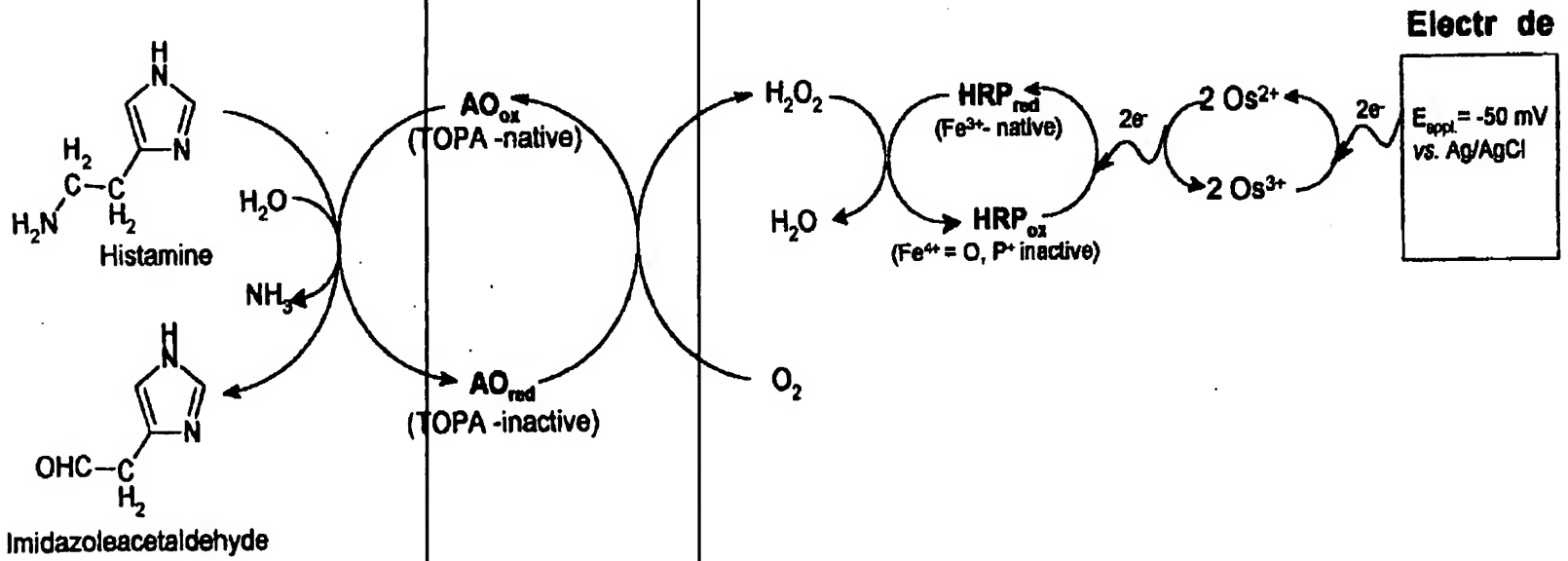
(42) Ohara, T. J.; Rajagopalan, R.; Heller, A. *Anal. Chem.* **1993,** *65,* 3512-3517.

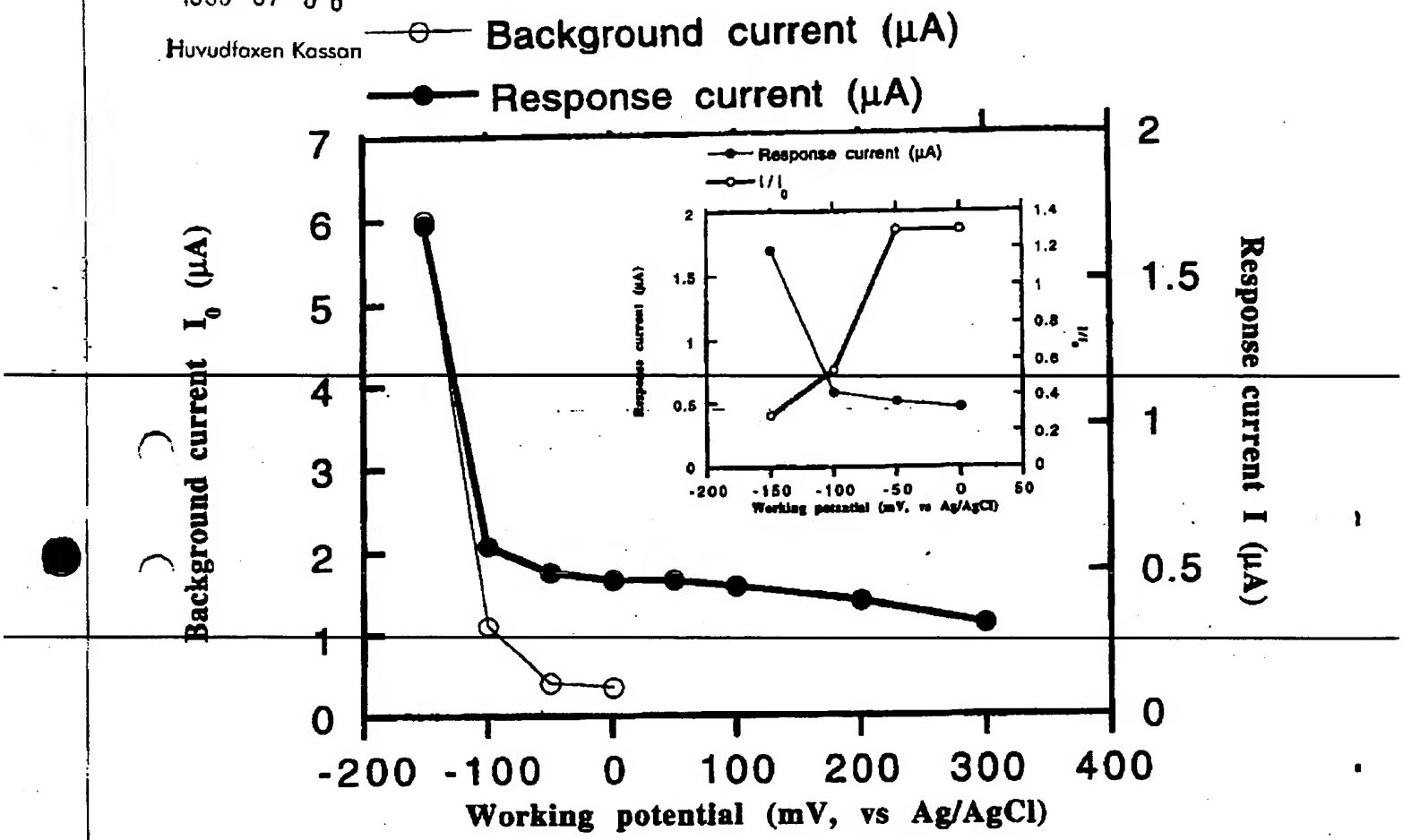
(43) Csöregi, E.; Schmidtke, D. W.; Heller, A. *Anal. Chem.* **1995,** *67,* 1240-1244.

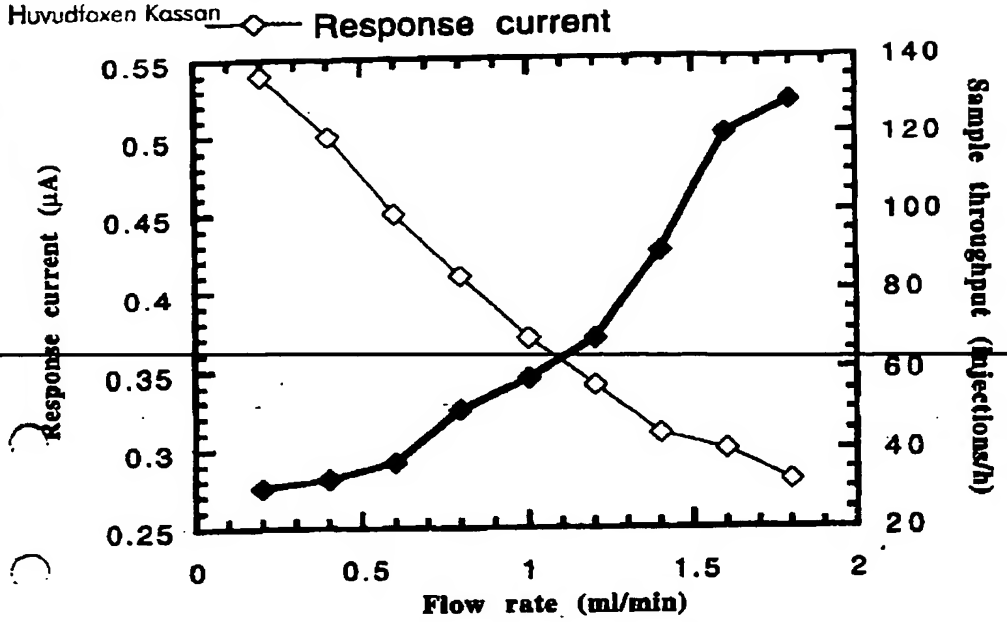
(44) Larsson, N.; Ruzgas, T.; Gorton, L.; Kokaia, M.; Kissinger, P. T.; Csöregi, E. *Electrochim. Acta* **1998**, *43*.

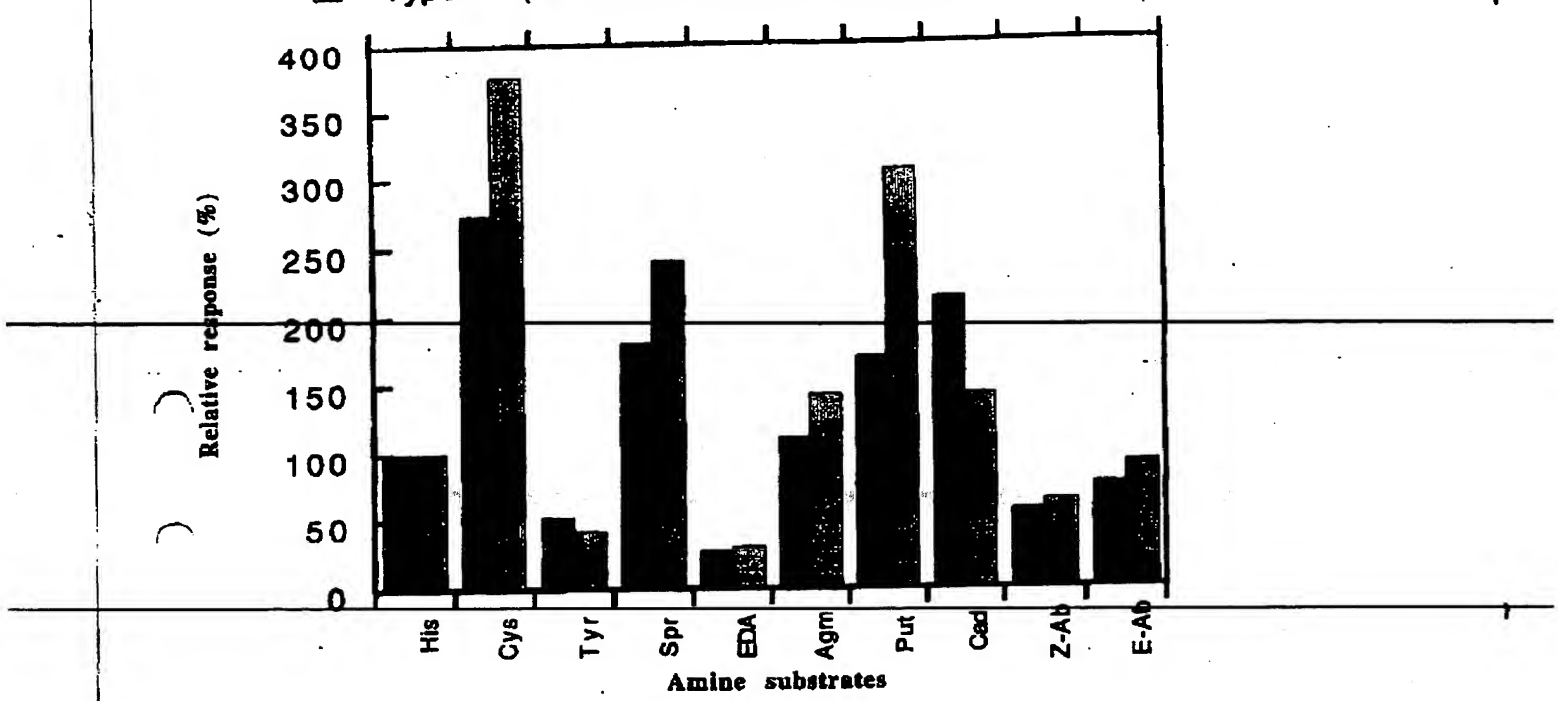
(45) Ohara, T. J.; Vreeke, M. S.; Battaglini, F.; Heller, A. *Electroanalysis* **1993**, *5*, 825-831.

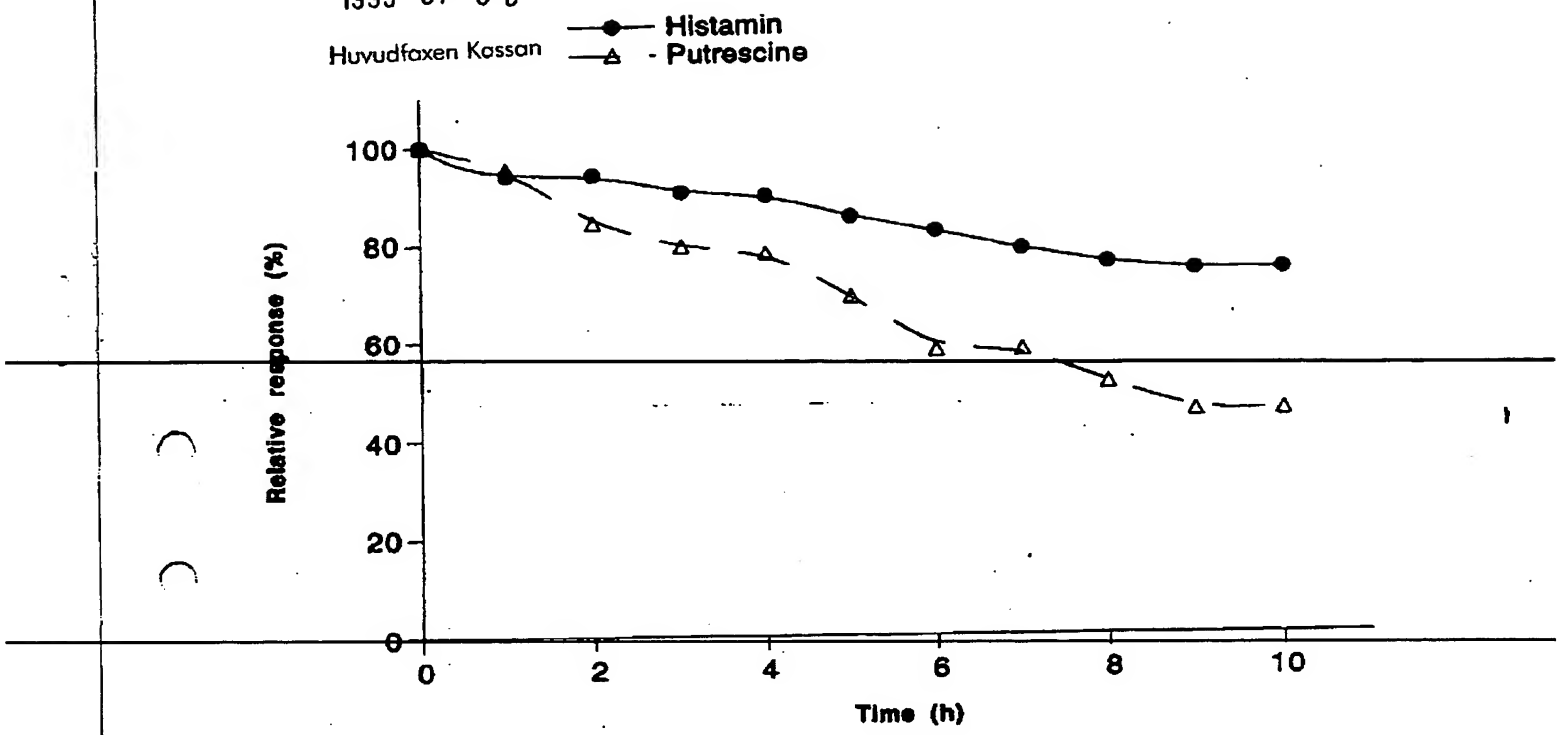




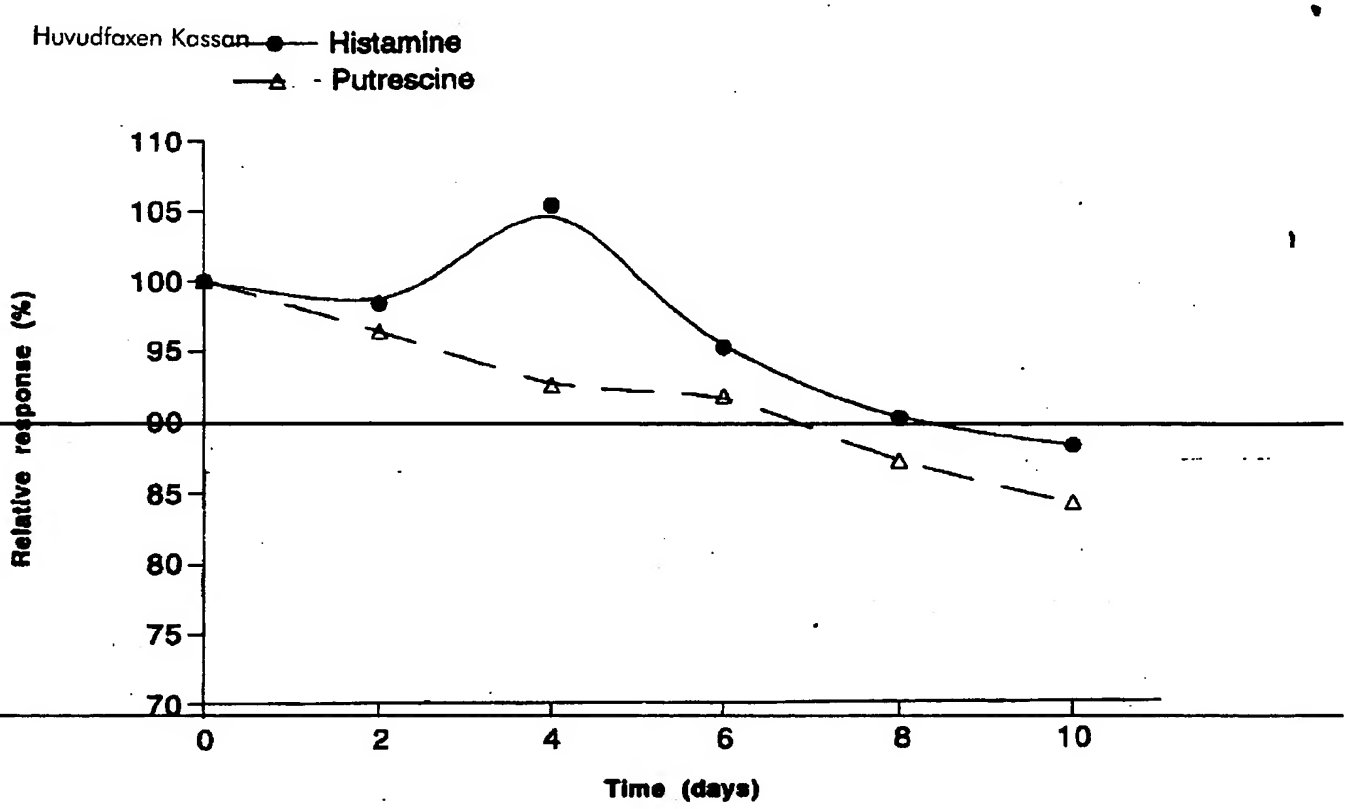








1999 -07- 0 6


Huvudfaxen Kassan
Histamine
Putrescine
110
105
100
95
90
85
80
75
70
Relative response (%)
0
2
4
6
8
10
Time (days)

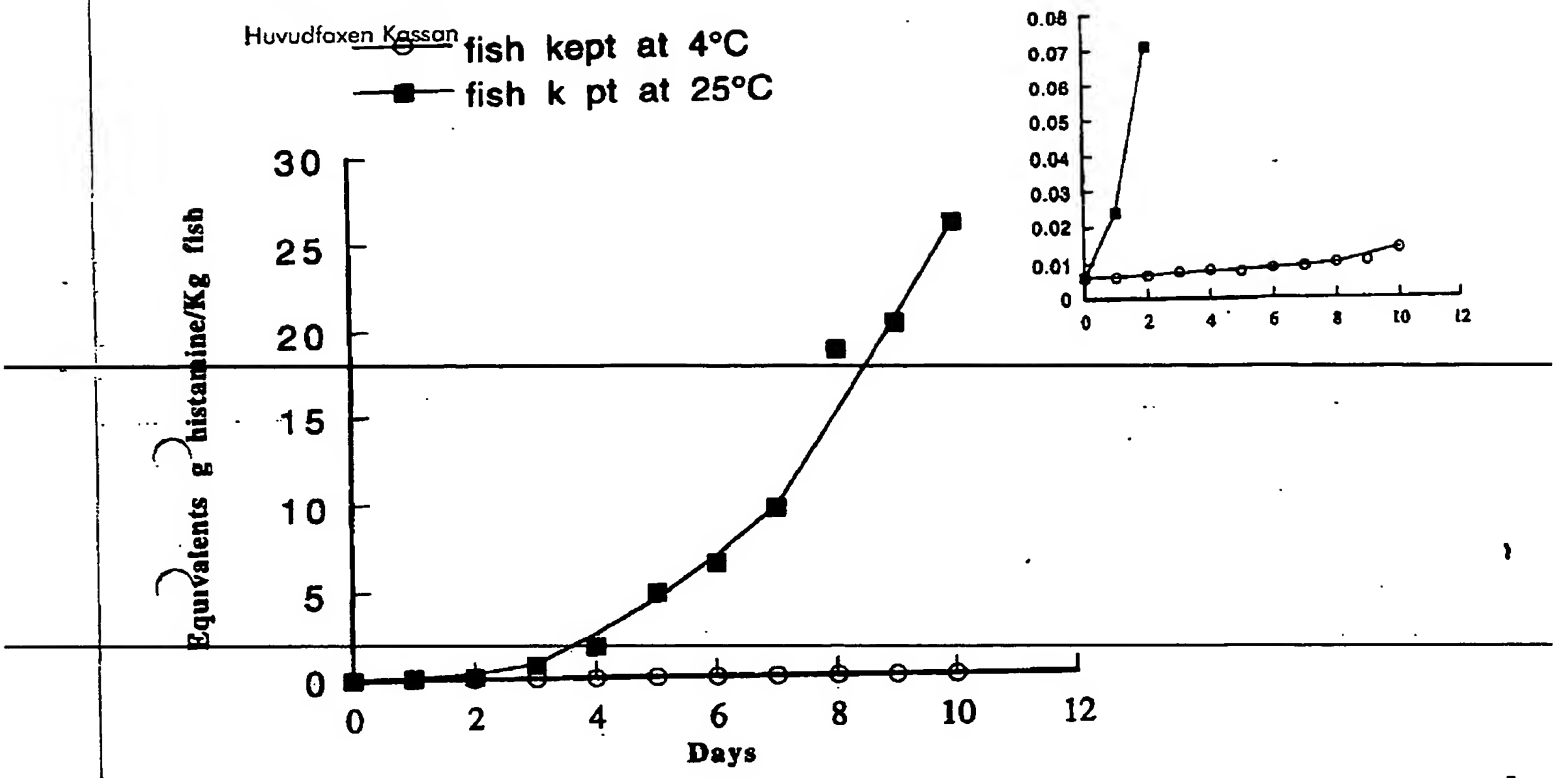


TABLE I: The modification of the apparent parameters together with the main biosensor characteristics with different AO:HRP ratios (w/w) immobilized on the surface of the working electrode.

Electrode type	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm²)	C (%) (S/S_{H2O2})	DL (μM)
AO 87%	Histamine	279±16	1.03±0.02	50.57±0.82	19.0	0.16
HRP 13%	Putrescine	153±15	1.96±0.06	175.48±1.40	66.2	0.06
	H_2O_2	93±3	1.80±0.21	265.13±1.65	-	-
AO 80%	Histamine	332±17	1.34±0.03	55.28±0.76	16.6	0.20
HRP 20%	Putrescine	228±15	3.01±0.07	180.84±0.95	54.7	0.07
	H_2O_2	112±8	2.70±0.06	330.23±1.02	-	-
AO 67%	Histamine	370±22	1.30±0.03	48.13±0.14	14.7	0.25
HRP 33%	Putrescine	240±15	3.10±0.01	176.94±0.87	54.2	0.07
	H_2O_2	153±6	3.64±0.04	325.90±0.56	-	-
AO 50%	Histamine	437±43	1.22±0.04	38.24±1.42	12.7	0.33
HRP 50%	Putrescine	268±23	3.05±0.10	155.90±1.26	52.0	0.08
	H_2O_2	175±8	3.83±0.05	299.80±0.65	-	-
AO 40%	Histamine	441±23	1.16±0.02	36.03±0.75	10.9	0.34
HRP 60%	Putrescine	276±22	3.69±0.06	183.14±1.11	55.7	0.13
	H_2O_2	206±3	4.94±0.03	328.50±0.22	-	-
AO 33%	Histamine	479±41	1.37±0.10	39.18±1.54	12.2	0.41
HRP 67%	Putrescine	287±12	3.84±0.06	183.28±0.61	57.0	0.08
	H_2O_2	211±18	4.95±0.15	321.36±1.24	-	-

where I_{max} and K_m^{app} values were estimated from the Michaelis-Menten equation:

$$I = (I_{max} \times [A])/(K_m^{app} + [A]),$$

and S - denotes sensitivity (calculated as I_{max}/K_m^{app}), C - conversion (calculated as $S_{analyte}/S_{H2O2}$), DL - detection limit (calculated as 3 S/N).

TABLE II. The effect of the electrochemical mediator and cross-linking agent concentrations, respectively, on the main biosensor parameters. DR is dynamic range and the others symbols are like in table I.

Electrode type	Analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/Mcm²)	C% $S/S_{H_2O_2}$	DL (μM)	DR (μM)
Type II	Histamine	332±17	1.34±0.02	55.29±0.73	16.74	0.16	1-100
	Putrescine	227±16	3.01±0.07	181.64±1.01	55.01	0.06	1-100
	H_2O_2	112±8	2.70±0.06	330.14±1.02			1-100
Type III	Histamine	901±85	4.85±0.41	73.74±1.73	23.07	0.33	1-150
	Putrescine	512±40	7.26±0.53	194.11±1.37	60.73	0.17	1-400
	H_2O_2	977±92	22.8±1.68	319.59±1.63			1-250

Abstract

This work reports the development and optimization of amperometric biosensors based on the enzyme amine oxidase (AO) for the detection of histamine, a well-known biomarker for food freshness.

Biosensor characteristics were evaluated in a flow injection (FI) analysis line, operated at +200 mV vs. Ag/AgCl (0.1 M KCl). Two different biosensor designs were considered. One is based on adsorbed AO on graphite electrodes, the detection being based on a direct electron transfer (DET) mechanism, whereas the second one is based on an Os-bipyridine modified redox polymer using a mediated electron transfer (MET) pathway. Both electrode designs were able to detect histamine in μM range, however [osmium(4,4'-dimethylbipyridine)$_2$Cl]$^{+/2+}$ complexed with poly(1-vinylimidazole) (PVI_{13}-dmeOs) based electrodes showed superior characteristics with regards to stability, selectivity and linear range.

MET-based electrodes were characterized by a detection limit of 2.2 μM (calculated as three times the signal-to-noise ratio), a sensitivity of 6.76 $mAM^{-1}cm^{-2}$, a dynamic range of 10-200 μM, and an operational stability of 20% response loss during 8h of continuous operation at a sample throughput of 30 injections h^{-1}.

Key words: histamine, amine oxidase, amperometric biosensor, flow injection.